

REMARKS

Claim Status

Upon entry of the amendments, claims 13, 16, 32, and 34-44 constitute the pending claims in the present application. Applicants have cancelled claims 45 and 46 without prejudice. Applicants reserve the right to pursue the subject matter of the cancelled claim and to pursue claims of similar or differing scope in future applications. Applicants respectfully request reconsideration in view of the following remarks. Issues raised by the Examiner will be addressed below in the order they appear in the prior Office Action.

Applicants have amended claim 13 to incorporate limitations similar to those of claim 45. Applicants have also amended claim 13 to recite “compared to a control cell.” Support may be found, for instance, in paragraphs 0071 and 0193 of the published application (U.S. Application Publication No. 20040063627). Furthermore, Applicants have amended claim 39 to incorporate the language of claim 46. The amendments are fully supported by the specification as filed. No new matter has been introduced and no new issue has been raised.

Claim rejections under 35 U.S.C. §112, second paragraph

Claims 13, 16, 32, and 34-46 are rejected under 35 USC 112, second paragraph, as allegedly indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention. Applicants respectfully traverse the rejection to the extent it is maintained over the claims as amended.

Specifically, the Office Action asserts that claim 13 is “vague and indefinite for recitation [of] ‘an abnormal dystrophin-associated complex (DAPC)’”. Applicants respectfully disagree. Nevertheless, to expedite prosecution, Applicants have incorporated a limitation similar to that of claim 45 into claim 13. Applicants note that the previous Office Action raised no objection to the language (i) in claim 45 “a mutation in a DAPC component”. However, the Examiner rejects the recitation (ii) in claim 45 “an abnormally low level of a DAPC component,” on the grounds that “the metes and bounds of the recitation can not be determined”. Solely to expedite prosecution,

Applicants have amended the recitation (ii) to recite "an abnormally lower level of a DAPC component compared to a control cell." Applicants believe that such amendment has obviated the rejection for the following reasons. First, Applicants note that relative language can be definite. Pursuant to MPEP 2173.05(b), "The fact that claim language, including terms of degree, may not be precise, does not automatically render the claim indefinite under 35 U.S.C. 112, second paragraph. Seattle Box Co., v. Industrial Crating & Packing, Inc., 731 F.2d 818, 221 USPQ 568 (Fed. Cir. 1984). Acceptability of the claim language depends on whether one of ordinary skill in the art would understand what is claimed, in light of the specification." Based on the state of the art at the time of filing, one of ordinary skill would encounter no difficulty in distinguishing between normal DAPC component levels and abnormally lower DAPC component levels compared to a control cell. One of skill in the art would recognize that an abnormally lower level of a DAPC component refers to a level sufficient to cause a pathology or a predisposition to a pathology. By way of non-limiting example, **Exhibits A-C** (Hoffman *et al.*, Cell. 1987 Dec 24;51(6):919-28; Matsumura *et al.*, Nature. 1992 Sep 24;359(6393):320-2; Mizuno *et al.*, Am J Pathol. 1995 Feb;146(2):530-6, respectively) disclose abnormally low levels of dystrophin, dystroglycan, and sarcoglycan in pathological tissue.

On page 3 of the Office Action, the Examiner states that incorporating claim 46 into claim 39 would obviate the rejection of claim 39. Applicants respectfully disagree with the rejection. Nevertheless, to expedite prosecution, Applicants have complied with the Examiner's suggestion.

Next, the Examiner rejects claim 13 for the recitation of "the cell-surface receptor muscle, skeletal receptor tyrosine kinase (MuSK)", stating "the metes and bounds of the recitation cannot be determined from the claim or the instant specification as filed." Applicants respectfully traverse. MuSK is the name of a particular kinase. "Muscle, skeletal receptor tyrosine kinase" is known in the art to be the full name of this kinase. MuSK is described in greater detail in **Exhibit D** (Glass *et al.* Cold Spring Harb Symp Quant Biol. 1996;61:435-44). Accordingly, the recitation "the cell-surface receptor muscle, skeletal receptor tyrosine kinase (MuSK)" is definite.

In light of the claim amendments and remarks above, reconsideration and withdrawal of this rejection is respectfully requested.

Claim rejections under 35 U.S.C. §102(b)

Claims 13, 16, 32, and 34-44 are rejected under 35 U.S.C. §102(b) as allegedly being anticipated by Ruoslahti *et al.*, US Patent No. 5,654,270. The Examiner maintains his previous grounds of rejection. Specifically, the Examiner takes the position that the phrase "wherein the cell has an abnormal dystrophin-associated protein complex (DAPC)" encompasses cells in a wounded tissue. This broad reading of the term "abnormal DAPC" is based on the current indefiniteness rejection. Applicants respectfully traverse the rejection. Specifically, Applicants believe that the remarks and amendments presented herein obviate the rejection. Ruoslahti does not teach administration of a biglycan therapeutic to a cell with abnormal DAPC "wherein the abnormal DAPC is caused by one or more of (i) a mutation in a DAPC component, or (ii) an abnormally lower level of a DAPC component compared to a control cell, wherein the DAPC component is: a dystroglycan, dystrophin, or a sarcoglycan," i.e., the subject matter of previously presented claim 45. In fact, the Examiner has acknowledged that claim 45 is novel over the cited reference. Applicants respectfully request reconsideration and withdrawal of the rejection.

Double Patenting Rejection

Claim 44 was rejected on the basis of alleged obviousness-type double patenting over claims 1-14 of U.S. Patent No. 6,864,236. Without conceding that the pending claims necessarily fully encompass the claims of the '236 patent, Applicants request that the Examiner hold the rejections made under the judicially created doctrine of obviousness-type double patenting in abeyance until otherwise allowable subject matter is identified in the instant application. Once allowable subject matter has been identified, Applicants will evaluate the filing of a terminal disclaimer or providing arguments in view of the claims pending at that time.

Conclusion

In view of the above amendment, Applicants believe the pending application is in condition for allowance.

Application No. 10/081,736
Amendment dated September 30, 2008
After Final Office Action of July 31, 2008

Docket No.: BURF-P02-006

Applicants believe no fee is due with this response. However, if a fee is due, please charge our Deposit Account No. 18-1945, under Order No. **BURF-P02-006** from which the undersigned is authorized to draw.

Dated:

September 30, 2008

Respectfully submitted,

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Dystrophin: The Protein Product of the Duchenne Muscular Dystrophy Locus

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(Dubowitz, 1985) and high concentrations of soluble muscle-specific enzymes in serum are present in affected individuals long before the clinical onset of the disease, and can often be found in female carriers (Emery and Holloway, 1977). Despite many years of intensive research, the primary biochemical defect responsible for this disorder has remained elusive, as have any rational therapies to slow the progression of the disease.

With no effective treatments available, an animal model for this disease has long been sought to test possible therapies. Despite the availability of numerous muscular dystrophies in many different species (Harris and Slater, 1980), the lack of any information concerning the biochemical defect involved in both DMD and the putative animal models has made it difficult to equate any specific animal muscular dystrophy with DMD. An X-linked murine muscular dystrophy, *mdx*, was fortuitously discovered during screening of normal mouse serum enzymes in preparation for a mutagenesis screen (Bulfield et al., 1984). Given the general conservation of the X chromosome within mammalian species (Ohno, Becak, and Becak, 1964), the chromosomal location of *mdx* in mouse suggested that the *mdx* mutation might indeed represent the same biochemical defect as that manifested in X-linked human DMD. However, homozygous *mdx* mice exhibit little, if any, clinically detectable phenotype. Homozygous strains are easily maintained, showing only slightly reduced fecundity (Torres and Duchen, 1987), and develop no obvious muscle weakness (Tanabe, Esaki, and Nomura, 1986). Histologically, the persistent degeneration/regeneration characteristic of human DMD muscle fibers appears very similar in the *mdx* mouse, though the extensive connective tissue proliferation (fibrosis) evident in human DMD muscle groups appears to be largely absent in *mdx* muscle (Bridges, 1986). Though it could be argued that the *mdx* mutation represents a mild allele of the mouse DMD homolog (such as the rarer Becker allele of Duchenne), two ethynitrosourea-induced (ENU) alleles of the *mdx* locus exhibit similar phenotypes, making it unlikely that all three represent Becker-like mutations (Chapman, personal communication).

The differences between the mouse *mdx* and human DMD phenotypes have been used as evidence against the significance of the common X-linked nature of the mutations. In apparent agreement with the phenotypic differences, the *mdx* mutation has been recently shown to be more closely linked genetically to markers neighboring the less severe and less common human X-linked Emery-Dreifuss muscular dystrophy (Avner et al., 1987). These two human X-linked dystrophies are on opposite ends of the human X chromosome, yet recent studies using portions of the cloned DMD locus as genetic markers in the mouse have placed the mDMD locus in the same chromosomal region as the *mdx* mutation (Chapman et al., 1985; Heilig et al., 1987; Brockdorff et al., 1987; Chamberlain et al., 1987).

Summary

The protein product of the human Duchenne muscular dystrophy locus (DMD) and its mouse homolog (mDMD) have been identified by using polyclonal antibodies directed against fusion proteins containing two distinct regions of the mDMD cDNA. The DMD protein is shown to be approximately 400 kd and to represent approximately 0.002% of total striated muscle protein. This protein is also detected in smooth muscle (stomach). Muscle tissue isolated from both DMD-affected boys and *mdx* mice contained no detectable DMD protein, suggesting that these genetic disorders are homologous. Since *mdx* mice present no obvious clinical abnormalities, the identification of the *mdx* mouse as an animal model for DMD has important implications with regard to the etiology of the lethal DMD phenotype. We have named the protein dystrophin because of its identification via the isolation of the Duchenne muscular dystrophy locus.

Introduction

The muscular dystrophies are a heterogeneous group of both human and animal hereditary diseases whose primary manifestation is progressive muscle weakness due to intrinsic biochemical defects of muscle tissue (Mastaglia and Walton, 1982). The most common and devastating of the human muscular dystrophies is the X-linked recessive Duchenne muscular dystrophy (DMD), first described in the mid-1800s (Meryon, 1852; Duchenne, 1868). Affecting approximately 1 in 3,500 boys, this genetic disorder exhibits no obvious clinical manifestation until the age of 3 to 5 years, when proximal muscle weakness is first observed. The ensuing progressive loss of muscle strength usually leaves affected individuals wheelchair-bound by the age of 11, and results in early death due to respiratory failure. Both the typical histological pattern of widespread degeneration and regeneration of individual muscle fibers in most skeletal muscle groups

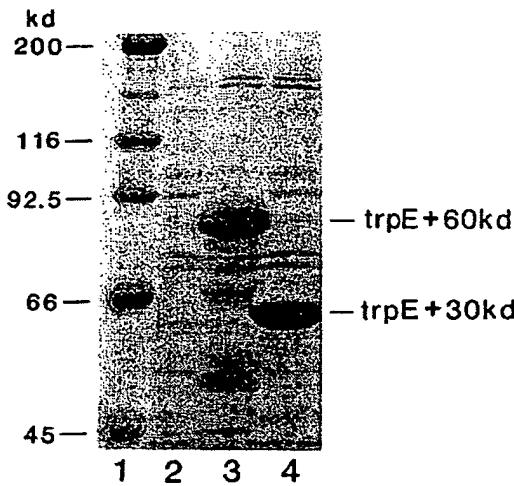


Figure 1. Insoluble Protein Fractions of Recombinant Bacterial Lysates

E. coli strain RR1 harboring either the parent plasmid vector (lane 2) (pATH2; Dieckmann and Tzagoloff, 1985) or one of its recombinants containing two different portions of the mDMD coding sequence (lane 3, trpE+60kd; lane 4, trpE+30kd) was induced with 3-B-indolacrylic acid and lysed, and insoluble proteins were isolated by centrifugation. Shown is 1% ($\sim 200 \mu\text{g}$) of the insoluble protein obtained from a 100 ml culture of bacteria for each preparation. The trpE-mDMD fusion proteins are evident as the major protein species ($\sim 85\%$ of total), at the expected molecular weights (trpE = $\sim 33 \text{ kd}$). Protein size markers are indicated (lane 1).

A human cDNA clone representing a portion of the DMD transcript has been isolated on the basis of its conservation between mouse and man (Monaco et al., 1986). This partial cDNA was then used to isolate homologous cDNA sequences from mouse (Hoffman et al., 1987), and the entire 14 kb human coding sequence (Koenig et al., 1987). The mRNA product of this locus was detected only in muscle-containing tissues in both humans and mice (Monaco et al., 1986; Hoffman et al., 1987), and has been demonstrated to be expressed specifically in terminally differentiated myotubes in culture (Lev et al., 1987). In this paper we report the production of antibodies to the mDMD and DMD gene protein products. We describe the use of these antibodies to study the protein product of the Duchenne muscular dystrophy locus, called dystrophin, in tissues isolated from both normal and dystrophic mice and humans.

Results

Production of mDMD Fusion Proteins

The DNA and predicted amino acid sequences for 4.3 kb (30%) of the Duchenne muscular dystrophy (DMD) gene has been previously presented for cDNA clones isolated from human fetal skeletal muscle and for the mouse cardiac muscle homolog of the human DMD locus (mDMD; Hoffman et al., 1987; Koenig et al., 1987). These cDNAs were found to be highly conserved between mice and humans, exhibiting over 90% similarity at both the DNA

and amino acid levels. To study the mouse and human protein product of the DMD locus, polyclonal antibodies were produced against large regions of the mouse mDMD polypeptide.

Two different regions of the mouse heart DMD cDNA were fused to the 3' terminus of the *E. coli* trpE gene by using the expression vector pATH2 (Dieckmann and Tzagoloff, 1985). The two regions represent the majority of the mouse heart cDNA sequence previously described (Hoffman et al., 1987), but do not overlap. One construction resulted in the fusion of approximately 30 kd of mDMD protein to the 33 kd trpE protein, while the second employed roughly 60 kd of the mDMD protein. Since the trpE protein is insoluble, quantitative yields of induced fusion proteins were obtained simply by lysing of the cells and precipitation of insoluble proteins. As shown in Figure 1, novel insoluble fusion proteins of the expected size were produced; they were not present in lysates from bacteria containing the pATH2 vector alone.

Production of Antisera

Both fusion proteins were purified by preparative SDS-polyacrylamide gel electrophoresis (Laemmli, 1970) and used to immunize rabbits and sheep (see Experimental Procedures). Rabbits were immunized with electroeluted, "native" (free from SDS) insoluble antigen, while sheep were immunized with SDS-polyacrylamide gel slices containing denatured antigen. In order to monitor the titer and specificity of immune sera, dot blots of each antigen were made on nitrocellulose by using the insoluble fractions of bacterial lysates harboring either the parent plasmid vector (pATH2) or a recombinant fusion-protein plasmid. Each antigen solution was loaded such that the amount indicated refers to the amount of trpE protein in each dot (Figure 2).

The DMD portion of the smaller fusion protein (trpE+30kd) proved to be highly antigenic. Greater than 95% of rabbit antibodies (rabbit serum; Figure 2A) were found to be directed specifically against the DMD portion of the polypeptide, though this portion represented only half of the fusion protein. Similarly, approximately 90% of the sheep antibodies were directed specifically against the DMD portion of this fusion protein (data not shown). The larger trpE+60kd fusion protein also proved to be quite antigenic, with a 1:1000 dilution of primary sera having antibody titers nearly equal to the limits of the detection system ($\sim 1 \text{ ng antigen}$), though a much larger proportion of antibodies were directed against the trpE protein (sheep serum shown in Figure 2B; rabbit serum results similar but not shown).

To ensure that any protein species identified by the antisera was due to recognition by antibodies specific for the DMD portion of the fusion proteins, rabbit and sheep antibodies directed against the 30 kd antigen and sheep antibodies directed against the 60 kd antigen were affinity-purified (Figure 2). Affinity purification of the rabbit antibodies directed against the mDMD portion of the fusion protein was facilitated by the insolubility of the partially purified fusion protein. By simple resuspension of crude insoluble protein fractions (shown in Figure 1) in immune se-

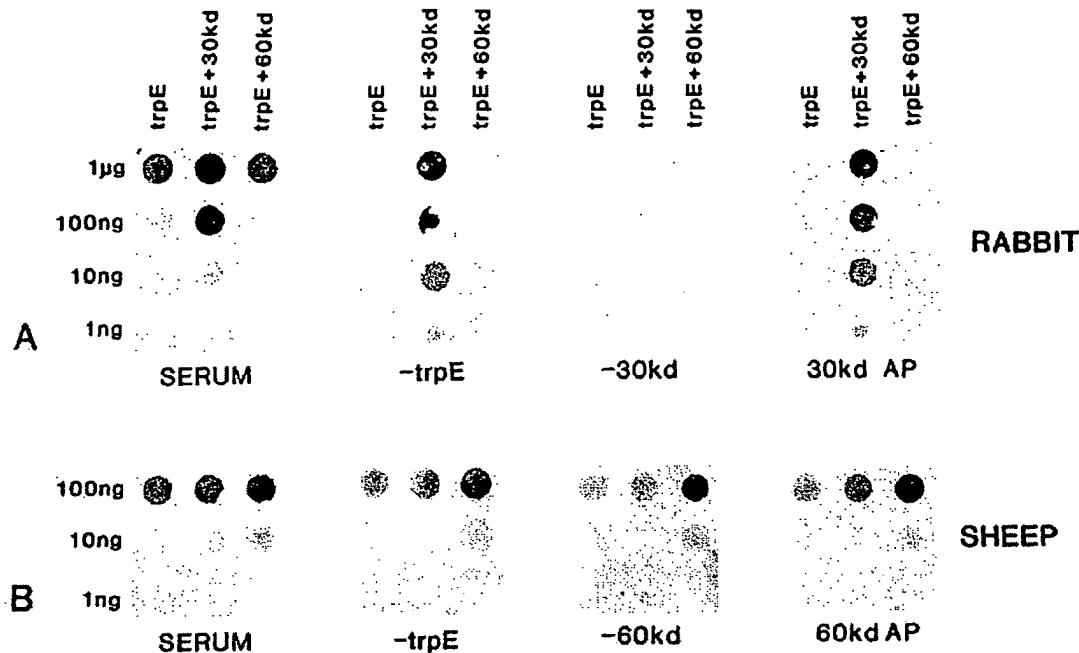


Figure 2. Specificity and Titer of Antisera Raised against the mDMD Fusion Proteins

Antigen dot blots were prepared on nitrocellulose by using the insoluble protein fractions shown in Figure 1. The amounts indicated refer to the relative amount of trpE protein in each dot.

(A) Immune serum from a rabbit immunized with the trpE+30kd antigen in insoluble form (see Experimental Procedures). The first filter shows the alkaline phosphatase staining after incubation with unprocessed immune serum diluted 1:1000, followed by incubation with alkaline phosphatase conjugated goat anti-rabbit second antibody (rabbit serum). Greater than 95% of the antibodies in the unprocessed serum are seen to be directed specifically against the DMD portion of the fusion protein. Antibodies specific for the trpE portion of the fusion peptide were then removed, with the resulting serum showing no apparent remaining reactivity for the trpE protein (-trpE). The immune serum with the antibodies against the 30 kd protein removed is shown to contain little remaining reactivity with the fusion protein (~30 kd). 30kd antigen-antibody complexes were dissociated, with the resulting supernatant showing a high titer of antibodies directed specifically against the DMD portion of the fusion peptide (30kd AP [affinity-purified]).

(B) The same affinity purification protocol as used in (A) was applied to immune serum from a sheep immunized with denatured trpE+60kd antigen.

rum, antibodies against the trpE protein were eliminated (-trpE; Figure 2A) and antibodies specific for the mDMD protein isolated (30kd AP). The immune serum that had been absorbed with both the trpE and trpE+30kd antigens showed very little remaining reactivity with either of these antigens (~30kd; Figure 2A). The resulting affinity-purified antibody (30kd AP) had a titer above the limits of the detection system (1 ng) when a 1:1000 dilution was used (Figure 2A). The sheep antisera against this same fusion protein were affinity-purified in the same manner, with greater than 95% of the resulting affinity-purified immunoglobulins being directed specifically against the 30 kd DMD antigen (not shown). In the case of the sheep antiserum directed against the 60 kd antigen, the same affinity purification protocol was used but appeared to be much less efficient (Figure 2B; -trpE, -60kd, 60kd AP).

Identification of the Protein Product of the Duchenne Muscular Dystrophy Locus

Total protein samples were isolated from mouse (fresh) and human (frozen) tissues by direct solubilization of tissues in 10 volumes of gel loading buffer (100 mM Tris, pH

8.0; 10% SDS; 10 mM EDTA; 50 mM DTT). Alternatively, Triton X-100 insoluble fractions were isolated from human and mouse tissues by homogenization in 0.25% Triton X-100 using a Waring blender at full speed, and by pelleting of insoluble proteins. The protein concentrations in the Triton-insoluble fractions were quantitated by using the Bio-Rad protein assay, while the protein concentration of the directly SDS-solubilized tissues was estimated based on the starting mass of the tissue used. All protein samples (50 µg) were separated by electrophoresis on 3.5% to 12.5% gradient SDS-polyacrylamide gels (Laemmli, 1970) using a 3.0% stacking gel, and transferred to nitrocellulose (Towbin, Staehelin, and Gordon, 1979). Identical nitrocellulose blots of the separated proteins were incubated with affinity-purified rabbit antibodies directed against the 30 kd antigen (Figure 3A), affinity-purified sheep antibodies directed against the 60 kd antigen (Figure 3B), and affinity-purified sheep antibodies directed against the 30 kd antigen (Figure 3C), each at a 1:1000 dilution. Immune complexes were detected by using either ¹²⁵I-protein A (Figure 3A) or alkaline phosphatase conjugated donkey anti-sheep IgG second antibody (Figures

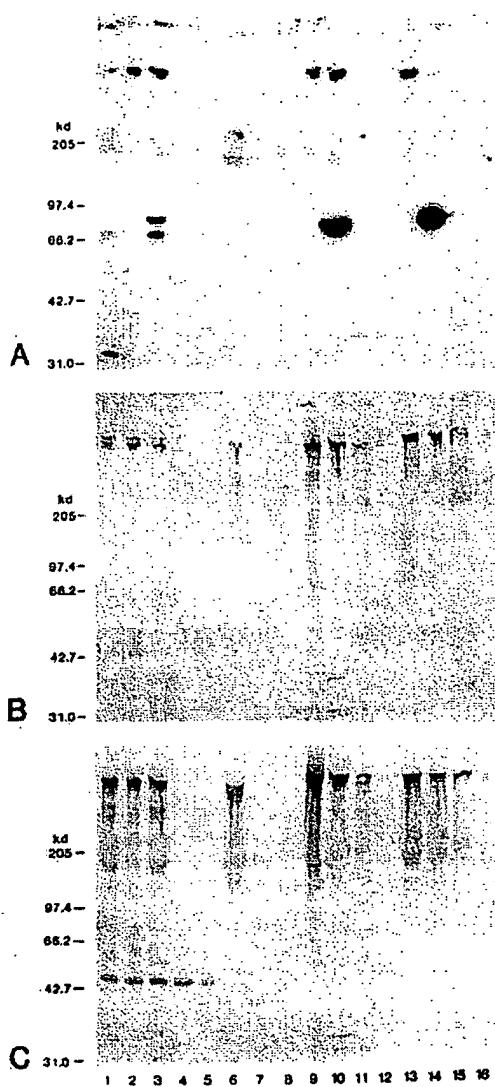


Figure 3. Identification of the Protein Product of the DMD Locus
 Mouse (fresh) or human (frozen) tissues were either directly solubilized and denatured in gel loading buffer or were first homogenized with 0.25% Triton X-100, and Triton-insoluble proteins were isolated. Protein samples (50 µg) were fractionated on 3.5%–12.5% gradient SDS-polyacrylamide gels (Laemmli, 1970), transferred to nitrocellulose (Towbin, Staehelin, and Gordon, 1979), and incubated with affinity-purified rabbit antibodies directed against the 30 kd cardiac mDMD (A), sheep antibodies directed against the 60 kd antigen (B), or sheep antibodies directed against the 30 kd antigen (C). Immune complexes were visualized by using either ^{125}I -protein A (A), or alkaline phosphatase conjugated donkey α -sheep IgG (B and C). Lanes are as follows: 1, human adult skeletal muscle; 2, human newborn cardiac muscle; 3, human newborn skeletal muscle (psosas); 4, human DMD-affected skeletal muscle (patient 1); 5, human DMD-affected skeletal muscle (patient 2); 6, Triton-insoluble extract of adult human skeletal muscle; 7, Triton-insoluble extract of DMD-affected skeletal muscle (patient 1); 8, Triton-insoluble extract of DMD-affected skeletal muscle (patient 2); 9, normal mouse heart; 10, normal mouse skeletal muscle; 11, normal mouse stomach; 12, normal mouse brain; 13, Triton-insoluble extract of normal mouse heart; 14, Triton-insoluble extract of normal mouse skeletal

3B and 3C) (sheep IgG binds very poorly to protein A). All antibodies detected a large molecular weight, apparently low abundance protein species calculated to be approximately 400 kd in total solubilized human and mouse skeletal and cardiac muscle (lanes 1–3, 9–10). The higher resolution of the alkaline phosphatase staining (Figures 3B and 3C) resolved this protein into doublets or triplets, though the slightly smaller bands most likely represent degradation products since there has been no evidence to date for alternatively spliced isoforms of the DMD mRNA (Koenig et al., 1987; Hoffman et al., 1987; Burghes et al., 1987). The 400 kd species was also clearly evident in mouse smooth muscle (stomach) (lane 11), though at a level substantially lower than that found in cardiac and skeletal muscle (lanes 9–10). The same apparent protein species was detectable in mouse brain at an extremely low level (Figures 3B and 3C, lane 12). Though transcriptional studies of the DMD gene in mice and humans (Monaco et al., 1986; Hoffman et al., 1987) were unable to identify DMD gene transcription in brain, the results presented here are completely compatible given the much greater sensitivity of the Western analysis used for protein detection relative to the Northern analysis used for mRNA detection of the large transcript. Further studies are required to determine whether the apparent low level of the DMD protein in brain is due to expression in smooth muscle or in other cell types.

The 400 kd protein species recognized by all antibodies was generally Triton-insoluble, though it appeared to be associated more strongly with the myofibrillar matrix fraction in cardiac muscle than it did in either skeletal muscle or smooth muscle (Figure 3, lanes 9–11, 13–15). This protein was not detectable in the SDS-solubilized (lanes 4–5) or Triton-insoluble (lanes 7–8) fractions of skeletal muscle samples from two boys affected with DMD. The deficiency of this protein in the DMD-affected boys is particularly evident in Figure 3C, where the presence of a cross-reactive 50 kd Triton-soluble protein serves to verify the equal protein content of the normal vs. DMD lanes (lanes 1–5).

Though sheep antibodies directed against the larger 60 kd DMD antigen (Figure 3B) recognized solely the 400 kd protein in both mouse and human tissues, additional protein species were clearly detected by rabbit and sheep antibodies raised against the 30 kd antigen (Figures 3A and 3C). However, none of the smaller proteins recognized by the sheep α -30kd antibodies were similarly recognized by the rabbit α -30kd antibodies, indicating that these smaller proteins represent cross-reactive protein species and are not themselves products of the DMD locus in either mice or humans. Though it appears that the amount of the DMD protein varies in different tissues depending on the anti-

muscle; 15, Triton-insoluble extract of normal mouse stomach; 16, Triton-insoluble extract of normal mouse brain.
 Shown is the 400 kd, low abundance protein species recognized by all three antibodies in all normal muscle-containing tissues. Smaller, cross-reactive protein species are detected by antibodies raised against the 30 kd DMD antigen in either rabbit (A) or sheep (C). The size and location of myosin and biotinylated molecular weight markers are indicated.

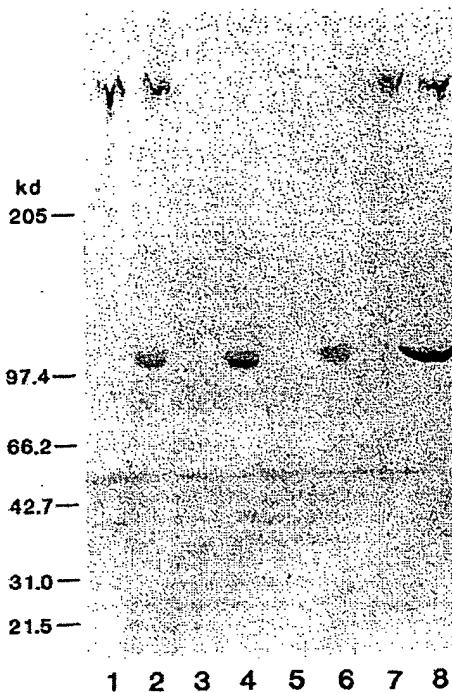


Figure 4. Analysis of *mdx* Mice

Freshly dissected heart and skeletal muscle tissues from normal, *mdx*, *mdx*⁴⁵⁷, and homozygous *Tr* mice were solubilized and denatured in gel loading buffer, and aliquots (50 µg) were fractionated on 3.5%–12.5% gradient SDS-polyacrylamide gels. Proteins were transferred to nitrocellulose and incubated with a mixture of rabbit (anti-30kd antigen) and sheep (anti-60kd antigen) anti-DMD antibodies, and the immune complexes were detected by using alkaline phosphatase conjugated second antibodies. Lanes are as follows: 1, homozygous *Tr* heart; 2, *Tr* skeletal muscle; 3, *mdx*⁴⁵⁷ heart; 4, *mdx*⁴⁵⁷ skeletal muscle; 5, *mdx* heart; 6, *mdx* skeletal muscle; 7, normal mouse heart; 8, normal mouse skeletal muscle.

The 400 kd protein species recognized by both sets of antibodies is evident in both normal and *Tr* mice, but is absent from both alleles of *mdx* mice. The more abundant 90 kd cross-reactive protein species recognized by rabbit antibodies directed against the 30 kd antigen (as shown in Figure 3A) is seen at equal levels in the skeletal muscle of all mice. This cross-reactive protein has an apparent molecular weight of 100 kd, which is probably a better representation of the size than the 90 kd weight calculated in Figure 3A. The size and location of myosin and biotinylated molecular weight markers are indicated.

body used (see Figure 3A vs. Figure 3B), this is assumed to be an artifact due to the increased contrast of the autoradiographic exposure of Figure 3A compared to the alkaline phosphatase staining of Figures 3B and 3C. Indeed, a comparison of Figures 3B and 3C, both of which employ immunochemical staining, indicates that antibodies raised against the two different antigens recognize the same 400 kd protein in equal relative abundances.

Analysis of *mdx* Mice

Skeletal and cardiac muscle was dissected from normal, *mdx* (Bullfield et al., 1984), *mdx*⁴⁵⁷ (V. Chapman, personal communication), and homozygous *Tr* (a severe neuro-

pathological disorder; Falconer, 1951; Henry, Cowen, and Sidman, 1983) mice and solubilized directly in gel loading buffer as described above. Protein samples were separated by electrophoresis on 3.5% to 12.5% gradient polyacrylamide gels and analyzed as above, by using a cocktail of sheep α -60kd and rabbit α -30kd antibodies. As shown in Figure 4, the 400 kd protein species was present in the skeletal and cardiac muscle of both normal (lanes 7–8) and *Tr* (lanes 1–2) mice. The detected protein appeared the same with this cocktail of antisera as it did with each antiserum separately (shown in Figure 3), indicating that the two antibodies raised against different antigens recognized the same protein. Both antibodies failed to detect the 400 kd protein in muscle tissues isolated from mice harboring either allele of the *mdx* mutation (lanes 3–6).

A much smaller skeletal muscle-specific cross-reactive polypeptide recognized by the rabbit antibodies raised against the 30 kd antigen (Figure 3A) appeared at equal levels in all mice, and serves to verify the quantity and quality of protein loaded in the skeletal muscle lanes. On the gradient gel in this experiment this smaller protein species has an apparent molecular weight of 100 kd, which probably represents a more accurate determination than that shown in Figure 3A.

Relationship between the DMD Protein and Nebulin

Nebulin, a large molecular weight, high abundance myofibrillar protein (Wang, 1985), has recently been implicated as being a candidate for the primary product of the DMD gene (Wood et al., 1987). To compare nebulin levels to those of the DMD protein, tissue samples from normal and DMD-affected human individuals and from normal, *mdx*, and *Tr* mice were directly solubilized in gel loading buffer, the proteins fractionated on 3.5% SDS-polyacrylamide gels, and the gels processed as above. Identical nitrocellulose blots were incubated with affinity-purified rabbit antibody directed against the 30 kd antigen (Figure 5A), or with guinea pig anti-rabbit nebulin (Figure 5B), followed by incubation with ¹²⁵I-protein A. As expected from the previous experiments, the anti-DMD antibodies recognized a 400 kd protein species in normal human skeletal muscle (Figure 5A, lanes 6 and 9) and in normal and *Tr* mouse skeletal and cardiac muscle (lanes 1–2, 10–11). This protein species was not detectable in human DMD muscle biopsies (Figure 5A, lanes 7–8), in either allele of *mdx* mouse (lanes 4–5, 12–13), or (in this experiment) in normal mouse brain (lane 3). The cross-reactive 100 kd skeletal muscle-specific protein species normally detected by the rabbit α -30kd antibodies used (see Figures 3A and 4) was run off the 3.5% gels, and is therefore not seen.

The anti-nebulin antibodies detected the expected abundant, skeletal muscle-specific protein species of approximately 500 kd (Wang, 1985; Hu, Kimura, and Maruyama, 1986; Locker and Wild, 1986), though this represents the first reported immunological evidence for the apparent absence of nebulin in cardiac muscle. Comparison of the autoradiographic exposure times and the signal intensities of the 400 kd DMD protein to those of

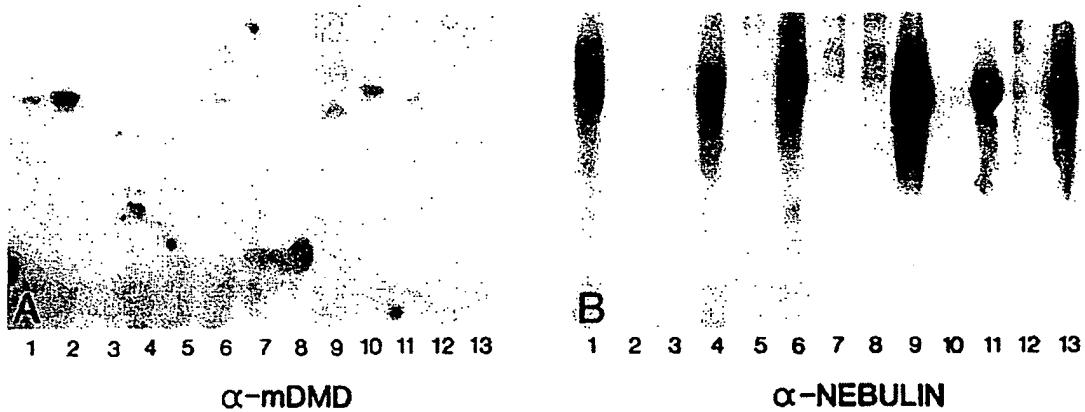


Figure 5. Comparison of DMD Protein Levels and Distribution with Those of Nebulin
 Mouse (fresh) and human (frozen) tissues were solubilized in sample loading buffer, with aliquots (50 µg) fractionated on 3.5% SDS-polyacrylamide gels. Proteins were transferred to nitrocellulose and incubated with antibodies directed against the 30 kd DMD antigen (A) or anti-nebulin antibodies (B), followed by detection of bound IgG with ^{125}I -protein A. Lanes are as follows: 1, normal mouse skeletal muscle; 2, normal mouse heart; 3, normal mouse brain; 4, *mdx* skeletal muscle; 5, *mdx* heart; 6, normal human skeletal muscle; 7, DMD skeletal muscle (patient 1); 8, DMD skeletal muscle (patient 2); 9, normal human skeletal muscle; 10, homozygous *Tr* heart; 11, *Tr* skeletal muscle; 12, *mdx*¹⁰⁷ heart; 13, *mdx*¹⁰⁷ skeletal muscle. The primary anti-DMD protein antibody and the detection system used for (A) is the same as for Figure 3A, and the levels and size of the DMD protein appear similar. The apparent size difference of dystrophin between lanes 9 and 10 in (A) is due to the larger amount of protein loaded in lane 9, such that the migration of the DMD protein is distorted by nebulin (B). In contrast to the DMD protein, nebulin appears as a larger (500 kd; Wang, 1985), more abundant, skeletal muscle-specific protein species in all mouse and human skeletal muscle samples tested. Autoradiography in (A) was for 3 days; (B), for 2 hr. Comparison of (A) and (B) shows that the DMD protein is approximately one-thousandth the level of nebulin.

nebulin indicated that the DMD protein was approximately one-thousandth the level of nebulin (Figure 5). Since nebulin has been calculated to represent about 3% of myofibrillar protein (and thus 1% of total muscle protein) (Wang, 1985), the DMD protein can be estimated to represent approximately 0.001% of total muscle protein.

Nebulin is evident, though greatly reduced, in the human DMD muscle samples tested (Figure 5B, lanes 7–8). On the other hand, nebulin appeared at normal levels in both alleles of *mdx* mouse (lanes 4–5, 12–13). This immunological data provides conclusive evidence that nebulin and the DMD protein are indeed distinct proteins, and therefore indicates that nebulin cannot be the protein product of the DMD locus.

Relative Cellular Abundance of the DMD Protein

The protein product of the DMD locus has been previously calculated to be in very low abundance on the basis of mRNA levels in muscle tissue (Hoffman et al., 1987). In order to measure more directly the cellular abundance of the DMD protein, the amount of this protein in heart was quantitated. Known quantities of DMD fusion proteins were denatured and fractionated on 3.5%–12.5% gradient SDS-polyacrylamide alongside 100 µg of solubilized mouse heart. Proteins were transferred to nitrocellulose, incubated with sheep antibodies directed against the 60 kd DMD antigen, and then detected by using alkaline phosphatase conjugated second antibodies. As shown in Figure 6, the antiserum reacts only with the antigen to which it was raised and not with the 30 kd fusion protein, indicating that all immunostaining is due to antibodies specific for the DMD portion of the trpE+60kd fusion pro-

tein. The signal exhibited for the 400 kd DMD protein in 100 µg of total cardiac protein corresponds to approximately 2 ng of the partially purified antigen to which the antibody was raised (Figure 6, lanes 3–4). Thus, by this measurement, the DMD protein comprises approximately 0.002% of total muscle protein.

Discussion

Recent reports have substantiated the correlation of recently described human cDNA sequences to the Duchenne muscular dystrophy locus in humans (Monaco et al., 1986; Monaco and Kunkel, 1987; Burghes et al., 1987; Koenig et al., 1987). Indeed, the fact that a large proportion of affected individuals exhibit small deletions within the genomic locus covered by cloned cDNAs indicates that these sequences represent the human DMD gene. The mouse homolog of the human DMD locus has been shown to reside on the mouse X chromosome by both genomic DNA analysis (Monaco et al., 1986) and cDNA analysis (Hoffman et al., 1987; Chamberlain et al., 1987; Heilig et al., 1987; Brockdorff et al., 1987). The human and mouse DMD cDNAs have been shown to be greater than 90% homologous over the entire amino-terminal one-third of the protein (~130 kd), diverging only upstream of their common translation initiation codon (Hoffman et al., 1987; Koenig et al., 1987). The predicted amino acid sequences of this portion of the human and mouse DMD proteins indicated that the protein might serve a highly conserved structural role in the myofiber (Hoffman et al., 1987). By the raising of antibodies to *in vitro* engineered fusion proteins containing portions of the mDMD protein (30 kd and

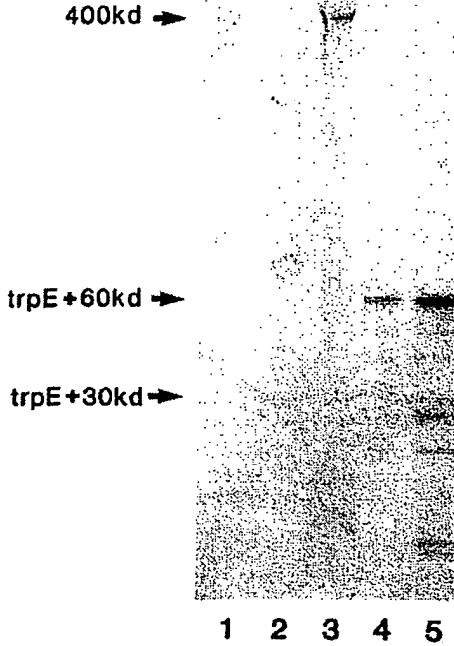


Figure 6. Quantitation of DMD Protein Levels in Normal Heart
Aliquots of bacterial lysates containing DMD-IrpE fusion proteins (see Figure 1) were quantitated, solubilized in gel loading buffer, and fractionated on 3.5%–12.5% SDS-polyacrylamide gels alongside 100 µg of solubilized mouse heart. Fractionated proteins were transferred to nitrocellulose and incubated with antibodies (sheep) directed against the 60 kd DMD antigen, followed by immune complex detection with alkaline phosphatase conjugated donkey anti-sheep second antibody. Lanes are as follows: 1, IrpE+30kd (2 ng); 2, IrpE+30kd (10 ng); 3, normal mouse heart (100 µg); 4, IrpE+60kd (2 ng); 5, IrpE+60kd (10 ng). The affinity-purified antibody directed against the IrpE+60kd antigen is seen to react specifically with this antigen and with the 400 kd DMD protein product. The signal intensity of the 400 kd DMD protein is seen to correspond to approximately 2 ng of partially purified antigen, indicating that the DMD protein represents approximately 0.002% of total heart protein. This calculation has an inherent uncertainty due to the variable transfer efficiencies of proteins of different sizes, though the antigenicity of these proteins should be identical because of their identical amino acid sequence.

60 kd), the protein product of the DMD locus was identified in both mouse and human muscle. The protein identified by these antibodies fulfills the following requirements expected of the primary gene product disrupted in DMD. First, the mRNA product of the mouse and human DMD loci has been estimated to be 16 kb in length (Monaco et al., 1986; Hoffman et al., 1987). This estimated size was revised to 14 kb with the complete cloning of the human cDNA (Koenig et al., 1987). The protein species described in this paper has been estimated to be approximately 400 kd, a size that is in general agreement with the translation of a mRNA of 14 kb. Second, the mRNAs corresponding to both the human and mouse DMD loci have been found to represent 0.01–0.001% of total muscle mRNA, as evidenced by both clone frequency in cDNA libraries and

abundance relative to α -tubulin mRNA (Hoffman et al., 1987). The abundance of the identified protein agrees with the mRNA abundance, as evidenced by abundance relative to nebulin (Figure 5) and direct quantitation in mouse heart (Figure 6). Third, the expression pattern of the mDMD gene has been studied at the mRNA level, with the large DMD transcript detectable only in striated and possibly smooth muscle (Monaco et al., 1986; Hoffman et al., 1987; Burghes et al., 1987). The 400 kd protein was clearly detected in skeletal and cardiac muscle, with smaller amounts in stomach (smooth muscle) (Figure 3). Fourth, the primary amino acid sequence of the amino-terminal 30% of the DMD protein has been shown to exhibit features common to many structural proteins, being highly conserved and rich in α -helix (Hoffman et al., 1987; Koenig et al., 1987). In agreement with this hypothesis, the DMD protein was found to be largely Triton-insoluble, suggesting an association with the myofibrillar matrix (Figure 3, lanes 13–15). Fifth, muscle biopsies from two DMD-affected individuals contained no detectable 400 kd protein (Figures 3 and 5), consistent with the molecular analysis of the DMD gene which has shown that most DMD-affected individuals possess null mutations of the DMD locus (Koenig et al., 1987). Sixth, antibodies raised in both rabbit and sheep against fusion peptides encoded by two separate, distinct regions of the DMD cDNA recognize the same protein species, as evidenced by the identical size, abundance, and tissue distribution of the detected protein. Taken together, this evidence validates the specificity of the described antibodies for the DMD gene product, and thereby substantiates the identification of this protein as the primary biochemical defect in Duchenne muscular dystrophy. Since we know of no previously reported protein that shares the abundance, sequence, or size characteristics of the DMD protein, and since this protein was identified by molecular genetic studies of patients affected with Duchenne muscular dystrophy, we have named this protein dystrophin.

It is interesting to note the very small amount of dystrophin present in total mouse brain tissue. A 30% incidence of mental retardation has been observed in boys afflicted with DMD (Zellweger and Hanson, 1967). It is tempting to speculate that the observed mental retardation could be a direct consequence of dystrophin absence, though the variable penetrance of this phenotype would argue against this. It is also interesting that, to date, both the mRNA and the protein have been found only in terminally differentiated cells (Hoffman et al., 1987; Lev et al., 1987; this paper). Given the greater than 2 million base pair size of the genomic DMD locus in humans (Monaco and Kunkel, 1987; Koenig et al., 1987), it would take more than 24 hr for RNA polymerase II to transcribe a single mRNA molecule from the DMD gene (Ucker and Yamamoto, 1984). If it is assumed that DNA replication and mRNA transcription cannot take place simultaneously, then only cells that are mitotically inactive for longer than 24 hr would be capable of transcribing the DMD gene, thus limiting the production of the DMD protein to predominantly mitotically inactive cells.

Our results concerning the absence of dystrophin in tis-

sues isolated from two alleles of *mdx* mice are particularly provocative. The lack of any detectable muscle weakness in *mdx* mice has led to the past hypotheses that the original *mdx* mutation represents either a mild allele of the mouse DMD homolog (Bulfield et al., 1984) or the homolog of the less severe human Emery-Dreyfuss dystrophy (Avner et al., 1987). We have found that two different mutant alleles of the *mdx* gene appear to lack the mDMD gene product, indicating that *mdx* and DMD most likely represent the same genetic disorder. Although the molecular defect in *mdx* has not been detected by DNA analysis (Chamberlain et al., 1987; L. M. Kunkel, unpublished data), the deficiency of dystrophin in *mdx* mice is presumed not to be a secondary consequence of a non-homologous genetic disorder for the following reasons. First, the homology of the *mdx* and DMD loci is consistent with the linkage data (Bulfield et al., 1984; Chapman et al., 1985; Heilig et al., 1987; Brockdorff et al., 1987; Chamberlain et al., 1987). Second, the deficiency of dystrophin in *mdx* mice appears to be disease-specific, as muscle samples from homozygous Trembler mice, afflicted with a severe neuropathological disorder (Henry, Cowen, and Sidman, 1983), exhibit wild-type levels of this protein. Third, though it could be argued that the absence of the mDMD product represents a generalized degradation of muscle proteins in *mdx* muscle, nebulin, which is regarded as one of the more labile muscle proteins (Wang, 1985; Sugita et al., 1987), is detectable at wild-type levels in *mdx* skeletal muscle (Figure 5B). Indeed, the normal levels of nebulin protein observed in mouse *mdx* contrasts to the severely reduced levels observed in human DMD patient muscle (Wood et al., 1987; Sugita et al., 1987). Such differences might be indicative of a more active role of endogenous proteases in human DMD muscle fibers, and could possibly explain some of the differences in the clinical phenotype.

By equating the mouse *mdx* and human DMD loci, an animal model is now available for DMD. The differences in the clinical manifestation of the same primary biochemical defect in mice and in humans might be explained by differences in secondary biochemical effects or histological changes. Histologically, both DMD and *mdx* muscle exhibit nearly identical patterns of myofiber degeneration and regeneration (Dubowitz, 1985; Bridges, 1986), a process that is probably a direct consequence of dystrophin deficiency in myofibers. The *mdx* muscle, however, never develops the extensive connective tissue proliferation (endomysial fibrosis) that is characteristic of human DMD muscle tissue, especially in the later stages of the disease (Dubowitz, 1985; Tanabe, Esaki, and Nomura, 1986; Torres and Duchene, 1987). This indicates that the prominent fibrosis in human DMD muscle is probably an indirect or secondary consequence of dystrophin deficiency. Perhaps the extensive endomysial fibrosis in human DMD muscles results in the impairment of the ability of individual muscle fibers to regenerate. This would mean that muscle fiber number would progressively decrease as the connective tissue content of each muscle group increases—a process that is, in fact, observed (Cullen and Fulthorpe, 1975; Watkins and Cullen, 1985). Such a process could ul-

timately result in insufficient muscle fiber numbers for mobility and respiration. The muscle fibers of *mdx* mice, on the other hand, exhibit no such fibrosis and retain the ability to regenerate throughout the life of the mouse, posing no threat to either mobility or normal life span. Possible rational therapies for boys afflicted with DMD might therefore result from the ability to control the connective tissue proliferation within the muscle tissue. Alternatively, future medical research could address the primary biochemical defect responsible for the DMD and *mdx* phenotypes, namely the deficiency of the dystrophin protein leading to fiber degeneration. Possible chemical agents that might result in a slowing of fiber degeneration could then be tested on *mdx* mice.

Conclusion

Molecular biological techniques have led to the identification of the primary biochemical defect in an important hereditary human disease, Duchenne muscular dystrophy. The identification of this defect was based solely on the chromosomal location of the DMD locus. The antibodies produced against the DMD protein product, dystrophin, should prove useful in the diagnosis and characterization of this disorder. As more is understood about the role of dystrophin in normal muscle function, rational therapies for the many boys affected with this fatal disease will, we hope, emerge. Many of these therapies could be tested on the *mdx* mouse model for this disease.

Experimental Procedures

Plasmid Constructions

The predicted amino acid sequence has been determined from the cDNA sequence for the amino-terminal one-third of the Duchenne muscular dystrophy gene product in both mice and humans (Hoffman et al., 1987; Koenig et al., 1987). Two different regions of the mouse sequence were fused to the *E. coli* *trpE* gene as follows, with the predicted number of amino acids being deduced from the DNA sequence.

trpE+60kd

The mouse DMD cDNA (Hoffman et al., 1987) was restricted at the unique *Spel* site, blunt-ended with Klenow, and then digested with *HindIII* in the 3' polylinker. The excised cDNA fragment of 1.4 kb was gel-purified and ligated to pATH2 (Dieckmann and Tzagoloff, 1985), which had been digested with *SmaI* and *HindIII*. Recombinants were identified by colony hybridization to random primer extended (³²P) insert (Feinberg and Vogelstein, 1983), and verified by subsequent plasmid DNA restriction analysis. The resulting plasmid construction fused the *trpE* protein (33 kd) to 410 amino acids (~60 kd) of the mDMD protein, and corresponds to position 1.3 kb to 2.7 kb on the equivalent human cDNA map (Koenig et al., 1987).

trpE+30kd

The most 3' end of the mouse cDNA currently available (Hoffman et al., 1987) was restricted at its unique nonmethylated *XbaI* site and at the *BamHI* site in the 3' polylinker. The excised 700 bp fragment was ligated to pPATH2 digested with *XbaI* and *BamHI* as described above. This plasmid construction fused the *trpE* protein to 208 amino acids (~30 kd) of the mDMD protein, and corresponds to position 3.7 kb to 4.4 kb on the equivalent human cDNA map (Koenig et al., 1987).

Induction and Purification of Fusion Proteins

Plasmid constructions were maintained in *E. coli* RR1, which was grown as suggested by A. Tzagoloff (unpublished data) except that 200 µg/ml of tryptophan was used as a supplement to all media. Induction with 3-B-indolacrylic acid (IAA), harvesting, and initial purification of *trpE* fusion proteins was as described by Dieckmann and Tzagoloff

(1985). Between 15 and 25 mg of insoluble protein was obtained from 100 ml of induced bacterial culture, of which approximately 85% was estimated to be the desired fusion protein (Figure 1). Between 2 and 5 mg of insoluble protein was solubilized by boiling in SDS, and then size-fractionated on preparative SDS-polyacrylamide gels (Laemmli, 1970). The fusion proteins were visualized by rinsing of the gels in distilled water for 5 min followed by immersion in cold 0.25 M KCl, with the appropriate protein band then being excised.

For rabbit immunizations, fusion proteins were then purified by electroelution into dialysis sacs, followed by precipitation with 5 volumes of acetone to remove SDS. Protein pellets, which also contained coprecipitated glycine, were resuspended in sterile 10 mM Tris (pH 8.0), and the protein concentration was determined (Bio-Rad protein assay on extensively sonicated aliquots).

For sheep immunizations, gel slices containing SDS-denatured antigen were sent to Polyclonal Seralabs (Cambridge, MA).

Antibody Production

New Zealand white female rabbits were immunized according to the following schedules: 1, intravenous injection (10 µg) with weekly boosts; 2, intradermal using 10 µg of fusion protein emulsified with Freund's complete adjuvant, with 10 µg boosts every 3 weeks using Freund's incomplete adjuvant; 3, intradermal as above (2) using 50 µg of fusion protein, with 100 µg boosts.

One rabbit was immunized with each fusion peptide according to each of the schedules. The titers and specificity of the antibodies produced in each rabbit were constantly monitored by enzyme-linked immunoassays performed on nitrocellulose dot blots of insoluble protein fractions such as those shown in Figure 2. The best immune responses were obtained by using the trpE+30kd polypeptide with immunization protocols 1 and 3 above, with >95% of the antibodies produced being specifically against the mDMD portion of the fusion peptide, and with titers greater than the sensitivity of the ELISA assay system when using a 1:1000 dilution of crude serum 4 weeks after immunization. The trpE+60kd antigen took much longer (12 weeks) to evoke an immune response in rabbits, with the resulting sera showing a low specificity for the DMD portion of the fusion protein (not shown).

A single sheep was immunized with each antigen in the form of SDS-denatured protein in polyacrylamide gel slices. Approximately 1 mg of fusion protein was used per immunization. The initial immunization was with Freund's complete adjuvant, with boosts using incomplete adjuvant at days 14 and 28. Injections were at multiple sites both intramuscularly and subcutaneously in lymph node areas. Serum was collected at day 50.

Antibody Purification

Approximately 3 mg of partially purified trpE protein (insoluble fraction; see above) was reprecipitated, resuspended in 10 mM Tris (pH 8.0), and then precipitated again. The pellet was resuspended in 1.5 ml of immune serum, incubated on ice for 1 hr, centrifuged to pellet the trpE-antibody immune complexes, and discarded. The supernatant was then mixed with approximately 3 mg of partially purified fusion protein (insoluble fraction) that had been washed as above. After incubation on ice, the mDMD-antibody immune complexes were precipitated by centrifugation. The pellet was then resuspended in 500 µl of 0.2 M glycine (pH 2.3), incubated on ice for 5 min to disassociate the immune complexes, and centrifuged at 4°C to precipitate the insoluble antigen. The supernatant containing the purified immunoglobulins was neutralized with 50 µl Tris (pH 9.5), and either stabilized with BSA (fraction V) (5 mg/ml) or dialyzed extensively against phosphate-buffered saline (PBS).

Western Blotting

Mouse (fresh) or human (frozen) tissues were homogenized in 10 volumes of gel loading buffer (Sugita et al., 1987) by using a motorized Teflon tissue homogenizer. The protein concentration of the solubilized tissues was approximated based on the weight of the tissues used. Mouse skeletal muscle samples used were total hind limb muscle.

Triton X-100 insoluble proteins were prepared by homogenization of fresh or frozen tissues in a Waring blender at high speed for 30 sec in buffer consisting of 10 mM HEPES (pH 7.2), 5 mM EGTA, 1 mg/ml PMSF, 1 mM iodoacetamide, 1 mM benzamidine, 0.5 mg/ml aprotinin, 0.5 mg/ml leupeptin, 0.25 mg/ml pepstatin A, and 0.25% Triton X-100.

Triton-insoluble proteins were precipitated and then resuspended in buffer without Triton X-100, and the protein concentration was determined (Bio-Rad protein assay). Aliquots were diluted with gel loading buffer, and 50 µg was used per lane. Protein samples were heated to 95°C for 2 min, centrifuged, and electrophoretically fractionated on 0.75 mm SDS-polyacrylamide gels (Laemmli, 1970), by using a 3% stacking gel and either a 3.5% or 3.5%-12.5% gradient resolving gel.

Fractionated proteins were transferred to nitrocellulose (Towbin, Staehelin, and Gordon, 1979), and the filters were dried. Dried filters were blocked in 5% nonfat dry milk in TBST (10 mM Tris, pH 8.0; 500 mM NaCl; 0.05% Tween-20). All immunological reagent dilutions and filter washes were done in TBST. The affinity-purified anti-mDMD antibodies, affinity-purified second antibodies (Sigma), and guinea pig anti-rabbit nebulin antisera were diluted 1:1000 in TBST prior to use. Affinity-purified ¹²⁵I-protein A was from Amersham, and was used at 5 uCi per 20 ml of TBST. Biotinylated molecular weight markers were purchased from Bio-Rad, and were visualized by using protocols and reagents supplied by the manufacturer. Myosin was easily visualized on all filters by immunostaining ghosts due to the abundance of this protein.

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The principal conclusion is that under circumstances where eye and head movements are generated unthinkingly, the two motor systems receive the same command at almost the same time. This seems to be the 'default' condition of the mechanism that directs our gaze. We can of course override it consciously by either making or suppressing head movements. Most of the time, however, the rules indicated here probably apply. □

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Deficiency of the 50K dystrophin-associated glycoprotein in severe childhood autosomal recessive muscular dystrophy

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X-LINKED recessive Duchenne muscular dystrophy (DMD) is caused by the absence of dystrophin, a membrane cytoskeletal protein^{1,2}. Dystrophin is associated with a large oligomeric complex of sarcolemmal glycoproteins³⁻¹⁰. The dystrophin-glycoprotein complex has been proposed to span the sarcolemma to provide a link between the subsarcolemmal cytoskeleton and the extracellular matrix component, laminin^{7,9}. In DMD, the absence of dystrophin leads to a large reduction in all of the dystrophin-associated proteins^{4,9,10}. We have investigated the possibility that a deficiency of a dystrophin-associated protein could be the cause of severe childhood autosomal recessive muscular dystrophy (SCARMD) with a DMD-like phenotype¹¹⁻¹⁴. Here we report the specific deficiency of the 50K dystrophin-associated glycoprotein (*M*, 50,000) in sarcolemma of SCARMD patients. Therefore, the loss of this glycoprotein is a common denominator of the pathological process leading to muscle cell necrosis in two forms of muscular dystrophy, DMD and SCARMD.

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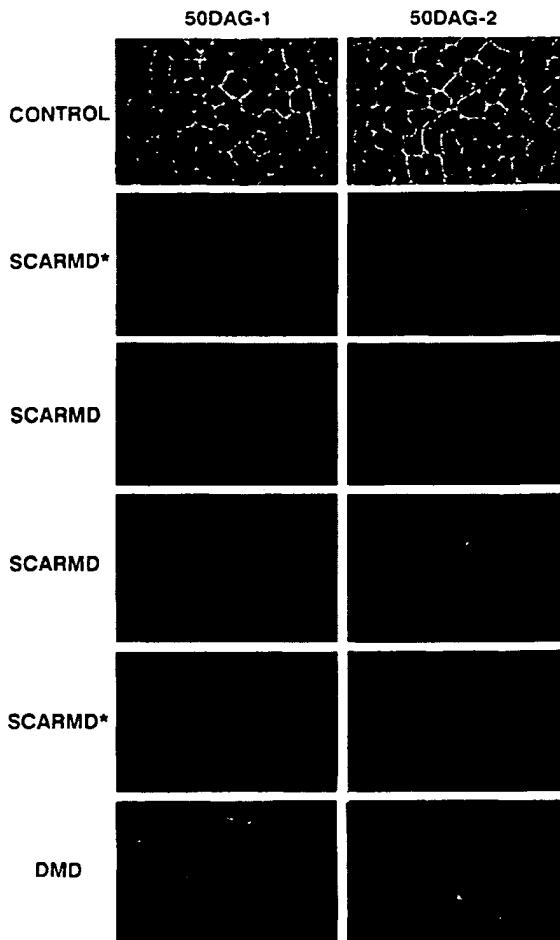


FIG. 2 Immunohistochemical analysis of 50DAG in biopsied skeletal muscle using a monoclonal antibody against 50DAG (50DAG-1) and a sheep polyclonal antibody affinity-purified against a 50DAG peptide (50DAG-2). Shown are (from top to bottom): 16-year-old male with no pathological changes in the skeletal muscle (CONTROL); 5-year-old male with SCARMD; 10-year-old female with SCARMD; 11-year-old male with SCARMD; 11-year-old female with SCARMD; and 8-year-old male with DMD (magnification, $\times 44$). *Siblings. METHODS. Serial transverse cryosections ($7 \mu\text{m}$) were immunostained with IVD3₁, a monoclonal antibody against 50DAG, and a sheep polyclonal antibody affinity-purified against 50DAG peptide as described previously⁴⁻¹⁰.

Figure 1 shows the immunohistochemical analysis of the muscle biopsy specimens using a monoclonal antibody against dystrophin and affinity-purified sheep polyclonal antibodies against 156K dystrophin-associated glycoprotein (156DAG), 59K dystrophin-associated protein (59DAP), 50K dystrophin-associated glycoprotein (50DAG), 43K dystrophin-associated glycoprotein (43DAG) and 35K dystrophin-associated glycoprotein (35DAG)⁴⁻¹⁰. In normal skeletal muscle, antibodies against dystrophin and dystrophin-associated proteins (DAPs) stained the sarcolemma. In addition, we have found no abnormality of these proteins in the following neuromuscular diseases: limb-girdle muscular dystrophy, myotonic dystrophy, oculopharyngeal muscular dystrophy, facioscapulohumeral muscular dystrophy, non-Fukuyama type congenital muscular dystrophy, congenital fibre type disproportion, spinal muscular atrophy and amyotrophic lateral sclerosis (not shown). In DMD patients, dystrophin was absent and the immunostaining for all of the DAPs was greatly reduced in the sarcolemma (Fig. 1), except at the neuromuscular junction and in the sarcolemma of intrafusal muscle fibres (data not shown). In contrast, in four

FIG. 1 Immunohistochemical analysis of dystrophin (DYS) and dystrophin-associated proteins in biopsied skeletal muscle. Shown are (from left to right): 16-year-old male with no pathological changes in the skeletal muscle (CONTROL); 5-year-old male with SCARMD; 10-year-old female with SCARMD; 11-year-old male with SCARMD; 11-year-old female with SCARMD; and 8-year-old male with DMD (magnification, $\times 28$). *, Siblings.

METHODS. Serial transverse cryosections ($7 \mu\text{m}$) were immunostained with VIA4₂, a monoclonal antibody against dystrophin, and affinity-purified sheep polyclonal antibodies against 156DAG, 50DAP, 59DAP, 43DAG and 35DAG as described previously⁴⁻¹⁰. The diagnosis of SCARMD was made on the basis of the following: (1) DMD-like phenotype affecting both males and females; (2) mode of inheritance compatible with an autosomal recessive disease; (3) North African patients; (4) elevated serum creatine kinase level; and (5) normal expression of dystrophin in the biopsied skeletal muscle analysed both by immunohistochemistry and by immunoblotting (Fig. 3)¹⁶.

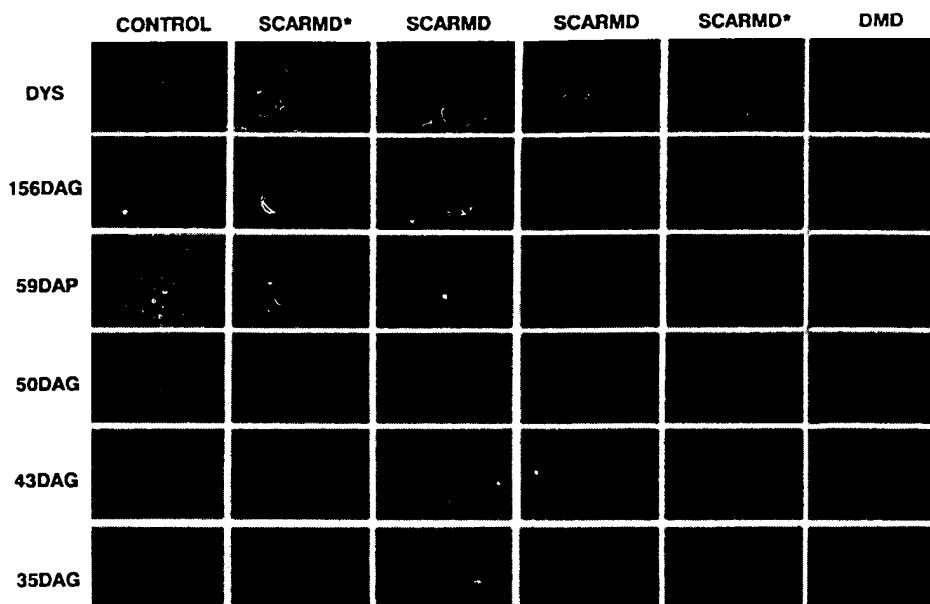
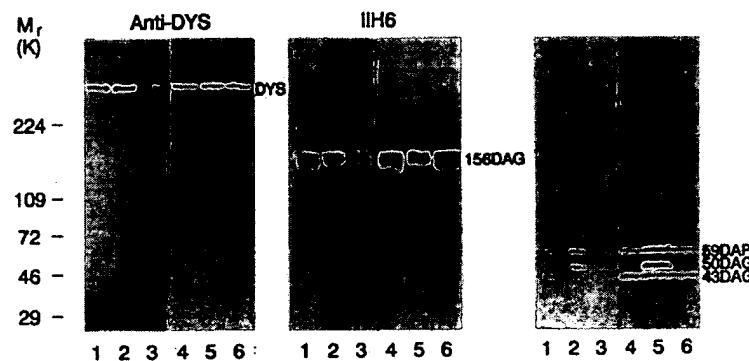


FIG. 3 Immunoblot analysis of the dystrophin (DYS)-glycoprotein complex in the SDS-extracts of the biopsied skeletal muscle. Shown are immunoblots stained with a polyclonal antibody against the last 10 amino acids of dystrophin (Anti-DYS), a monoclonal antibody against 156DAG (IIH6) and a cocktail of affinity-purified sheep polyclonal antibodies against 59DAP, 50DAG and 43DAG (Anti-DAPs), respectively. The affinity-purified sheep polyclonal antibody against 35DAG was not strong enough to stain the 35DAG in crude muscle extracts. Lanes 1 to 6 are samples from: 5-year-old male with SCARMD; 41-year-old female with facioscapulohumeral muscular dystrophy; 10-year-old female with SCARMD; 11-year-old male with SCARMD; 16-year-old male with no pathological changes in the skeletal muscle; and 11-year-old female with SCARMD (sister of patient in lane 1), respectively. Molecular weight standards ($\times 10^{-3}$) are shown on the left. **METHODS.** Cryosections ($20 \mu\text{m}$) from skeletal muscle biopsy specimens were homogenized in 50 vols of SDS-extraction buffer (80 mM Tris-HCl, pH 6.8, 10% SDS, 0.115 M sucrose, 1% β -mercaptoethanol, 1 mM PMSF, 1 mM benzamidine and 1 mM EDTA) and incubated at 50 °C for 10 min. After centrifugation, 10- μl samples were separated on 3-12% SDS-PAGE. The gel was stained with Coomassie blue and the density of the myosin



heavy chain band was measured using a computing laser densitometer (model 300S; Molecular Dynamics, Sunnyvale, CA). On the basis of this result, samples were run on 3-12% SDS-PAGE so that the amount of myosin heavy chain was equal for all specimens. Transfer to nitrocellulose membrane and immunostaining with antibodies were done as described³⁻¹⁰.

(Fig. 3). The affinity-purified antibody against the 35DAG was not strong enough to stain the 35DAG in crude muscle extracts. The deficiency of the 50DAG in SCARMD muscle extracts was confirmed using two other antibodies against the 50DAG (data not shown).

SCARMD (MIM number 253700)¹¹ is a progressive muscular dystrophy prevalent in North Africa¹¹⁻¹⁴. This disease shares several clinical features with DMD: mode of onset, rapid progression, hypertrophy of calves and extremely high serum creatine kinase levels in the initial stages of the disease. Dystrophin and dystrophin-related protein, an autosomal homologue of dystrophin¹⁵, are expressed normally in skeletal muscle in this disease^{13,16}. The structure and function of the dystrophin-glycoprotein complex (DGC) as a transsarcolemmal linker between the subsarcolemmal cytoskeleton¹⁷ and the extracellular component, laminin^{7,9}, suggest that a deficiency of a DAP could be the cause of an autosomal recessive muscular dystrophy with a DMD-like phenotype. Here we have demonstrated the specific deficiency of the 50DAG in the

patients with severe childhood autosomal recessive muscular dystrophy (SCARMD), including two siblings, immunostaining for the 50DAG was drastically diminished in the sarcolemma of all muscle fibres, including the neuromuscular junction and the sarcolemma of intrafusal muscle fibres, whereas immunostaining for dystrophin, 156DAG, 59DAP and 43DAG was preserved. Loss of 50DAG in SCARMD patients was more severe than in DMD patients. Immunostaining for 35DAG in SCARMD was reduced compared with normal control but was not as severely reduced as in DMD patients. Loss of 50DAG in the sarcolemma of SCARMD patients was confirmed using three other specific antibodies against the 50DAG, a monoclonal antibody (Fig. 2), a sheep polyclonal antibody affinity-purified against a 50DAG peptide (Fig. 2) and an affinity-purified guinea-pig polyclonal antibody (data not shown)⁴⁻¹⁰.

To confirm the deficiency of the 50DAG in SCARMD, skeletal muscle biopsy extracts were analysed by immunoblotting. Although dystrophin, 156DAG, 59DAP and 43DAG were detected, 50DAG was undetectable in all four SCARMD patients

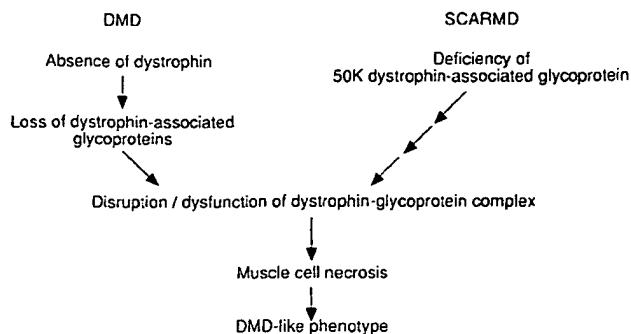


FIG. 4 Hypothetical scheme on the mechanism of muscle cell necrosis in severe childhood muscular dystrophies.

SCARMD sarcolemma. In the DMD sarcolemma, in contrast, the absence of dystrophin leads to a large reduction in all of the DAPs, including the 50DAG (Fig. 1, DMD panel)^{4,9,10}. Our results indicate that the loss of the 50DAG in the sarcolemma is a common denominator of the pathological process that leads eventually to muscle cell necrosis in two forms of muscular dystrophy, DMD and SCARMD.

On the basis of the function of the DGC mentioned above, we propose a hypothesis on the molecular mechanism of muscle cell necrosis in these two diseases where the disruption/dysfunction of the DGC plays a key role in the cascade of events leading to muscle cell necrosis (Fig. 4). In DMD, the absence of dystrophin leads to the loss of all DAPs, causing the disruption of the link between the subsarcolemmal cytoskeleton and the extracellular matrix that leads to sarcolemmal instability^{4,8-10}. In the case of SCARMD, in contrast, the deficiency of the 50DAG may severely disturb the function of the DGC and/or destabilize the DGC, also leading to sarcolemmal instability, which eventually causes muscle cell necrosis (Fig. 4).

The moderate reduction of the 35DAG in SCARMD could be secondary to the loss of the 50DAG. The 50DAG and 35DAG may form a tight subcomplex in the DGC and the loss of the 50DAG may cause a secondary reduction of the 35DAG in SCARMD. Dystrophin and 156DAG appeared reduced in the immunoblot analysis of the muscle specimen from the SCARMD patient with the most severe phenotype (lane 3 in Fig. 3). This suggests that the other components of the DGC could also be affected in the advanced stages of the disease.

Our results demonstrate the specific deficiency of the 50DAG in the SCARMD sarcolemma, leading to the reasonable hypothesis that this deficiency is causative of the disease. At present, the primary defect causing this deficiency is not known. It could be caused by a primary defect in the structure or expression of the gene for the 50DAG or could be due to a secondary effect of an unknown primary defect. Molecular biological and linkage analysis will be needed for the elucidation of the primary cause of SCARMD. But our results demonstrate that the diagnosis of SCARMD is now already possible by the immunochemical analysis of the 50DAG in muscle biopsy specimens. This will greatly aid the differential diagnosis between DMD and SCARMD. □

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Amyloid β -peptide is produced by cultured cells during normal metabolism

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ALZHEIMER'S disease is characterized by the extracellular deposition in the brain and its blood vessels of insoluble aggregates of the amyloid β -peptide ($A\beta$), a fragment, of about 40 amino acids in length, of the integral membrane protein β -amyloid precursor protein (β -APP)¹. The mechanism of extracellular accumulation of $A\beta$ in brain is unknown and no simple *in vitro* or *in vivo* model systems that produce extracellular $A\beta$ have been described. We report here the unexpected identification of the 4K (M_r , 4,000) $A\beta$ and a truncated form of $A\beta$ (~3K) in media from cultures of primary cells and untransfected and β -APP-transfected cell lines grown under normal conditions. These peptides were immunoprecipitated readily from culture medium by $A\beta$ -specific antibodies and their identities confirmed by sequencing. The concept that pathological processes are responsible for the production of $A\beta$ must now be reassessed in light of the observation that $A\beta$ is produced in soluble form *in vitro* and *in vivo*² during normal cellular metabolism. Further, these findings provide the basis for using simple cell culture systems to identify drugs that block the formation or release of $A\beta$, the primary protein constituent of the senile plaques of Alzheimer's disease.

Human kidney 293 cells stably transfected with complementary DNA encoding the 695-amino-acid isoform of β -APP³ were metabolically labelled with ^{35}S -methionine. Immunoprecipitation of the conditioned medium with R1280, a high-titre anti-serum against the synthetic peptide corresponding to residues 1-40 of $A\beta$ ($A\beta_{1-40}$; ref. 4), followed by electrophoresis and fluorography revealed two low-molecular-weight proteins migrating at ~4K and ~3K (Fig. 1a). The 4K band comigrated with radioiodinated synthetic $A\beta_{1-40}$. The 4K and 3K bands were absent in precipitations with preimmune serum or R1280 preadsorbed with synthetic $A\beta_{1-40}$ (Fig. 1a, b). Two additional high-titre polyclonal $A\beta$ antibodies also precipitated the 4K peptide before but not after adsorption with $A\beta_{1-40}$ (Fig. 1a). Medium from β -APP₆₉₅-transfected 293 cells contained substantially more of both the 4K and 3K peptides and the 90-100K soluble β -APP derivative (APP_s ; refs 5-8) than did medium from untransfected cells (Fig. 1b, c). In contrast, none of these

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Sarcoglycan Complex Is Selectively Lost in Dystrophic Hamster Muscle

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We recently reported that the dystrophin-associated glycoprotein (DAG) complex is biochemically divided into two subcomplexes: one is the dystroglycan complex comprised of 156DAG and 43DAG and the other is the sarcoglycan complex comprised of 50DAG, A3b, and 35DAG. A3b is a novel dystrophin-associated glycoprotein with an approximate molecular mass of 43 kd but is distinct from 43DAG. In the present study, we examined the striated muscles of the dystrophic hamster with anti-A3b antibody in addition to anti-50DAG, anti-43DAG, anti-35DAG, anti-dystrophin, and anti-laminin antibodies by both immunobistochemistry and immunoblot analysis and found that 50DAG, A3b, and 35DAG are selectively lost. This selective defect of the sarcoglycan complex in dystrophic hamster muscles may give rise to dystrophic changes in striated muscles. Thus, the differentiation of the dystrophin-associated glycoprotein complex into the dystroglycan and sarcoglycan complexes is important not only from a biochemical standpoint but also in understanding the cause of muscular dystrophy in the hamster. Our findings further show that the dystrophic hamster may serve as an animal model for a human disease, severe childhood autosomal recessive muscular dystrophy, which has recently been shown to result from a selective defect in the sarcoglycan complex. (Am J Pathol 1995; 146:530-536)

Dystrophin, a loss of which causes Duchenne muscular dystrophy (DMD),^{1,2} is present in normal muscle at the inner surface of the sarcolemma³ and is asso-

ciated with actin filament⁴ and dystrophin-associated proteins (DAPs)⁵ at its amino- and carboxyl-terminal regions, respectively. DAPs are classified into membrane-integrated and nonintegrated proteins.^{6,7} The former are the membranous dystrophin-associated glycoproteins (DAGs) that are known to exist in the large oligomeric glycoprotein complex^{6,8} and the latter are the peripheral proteins,^{6,7} that is, α -⁹ and β 1-syntrophin¹⁰ (collectively 59DAP) and A0.

Ervasti et al¹¹ and Ohlendieck et al¹² reported that all DAPs are greatly reduced by up to 90% in amount in DMD muscles. In contrast to these results, we found that in DMD muscle, although 43DAG is decreased in amount it is still fairly well preserved,^{13,14} whereas 50DAG and 35DAG are decreased to a greater degree than 43DAG,^{14,15} showing that the extent of reduction differs depending on the glycoprotein. Matsumura et al¹⁶ observed that a deficiency of 50DAG and a reduction of 35DAG give rise to a DMD-like disease called severe childhood autosomal recessive muscular dystrophy (SCARMD). These observations suggest that a loss of certain DAPs can cause dystrophic changes in muscle fibers. Meanwhile, Roberds et al¹⁷ reported that 43DAG is present in apparently equal amounts in control and dystrophic hamster skeletal muscles whereas 50DAG is undetectable and 35DAG appears to be slightly decreased in dystrophic muscle. Their findings differ slightly from ours.¹⁸ We found that, in dystrophic hamster skeletal muscle, not only 50DAG but also 35DAG is greatly reduced whereas 43DAG is present at an almost normal level.¹⁸ There are qualitative differences in the composition of dystrophin-associated glycoprotein complex. We found that 43DAG is ubiquitously present in a variety of tissues¹⁹ whereas 50DAG and 35DAG are present only in striated muscles.^{15,19}

Very recently, by gel filtration after treatment of the purified dystrophin-DAP complex with octyl β -D-

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glucoside²⁰ we found that the glycoprotein complex is divided into two subcomplexes. One complex consists of 156DAG and 43DAG, which we named the dystroglycan complex after Ibraghimov-Beskrovnyaya et al²¹ who reported that both glycoproteins are encoded by a single mRNA and then derived post-translationally from a single precursor protein, dystroglycan.²¹ Our report shows that these glycoproteins bind to form a complex after post-translational processing. The other, composed of 50DAG, A3b, and 35DAG, was named the sarcoglycan complex by us²⁰ because of its selective presence in striated muscles, although we did not determine the tissue distribution of A3b. A3b was originally described as being the smaller component of the 43DAG doublets^{8,13} but was recently found to be a novel DAG distinct from 43DAG.²⁰ However, its relation to dystrophic changes of the muscle fiber has not yet been elucidated.

Purified A3b was obtained by the gel filtration method mentioned above, which allowed us to raise a polyclonal antibody against it.²⁰ In this study, we examined whether structural defects in the glycoprotein complex are involved in the dystrophic changes in hamster skeletal and cardiac muscles. We found that the defect in the dystrophic hamster is mainly confined to the sarcoglycan complex and that dystrophin, 43DAG, and laminin are well preserved. Therefore, it is tempting to speculate that the gene defect in the dystrophic hamster may be localized to one of the genes coding for 50DAG, A3b, or 35DAG, which are the components of the sarcoglycan complex, and that the loss of the entire complex may result from the absence of only one of its components.

Materials and Methods

Muscles

Skeletal and cardiac muscles of dystrophic hamsters aged 6 to 12 months introduced from the BIO14.6 to the NSJ strain (NSJ-my/my) and control hamsters (NSJ-+/+) were examined.

Antibodies

Monoclonal anti-dystrophin antibody (DY2,²² Novo-castria Inc., Newcastle, UK), monoclonal anti-35DAG antibody (MA4-2¹⁵), and four polyclonal antibodies, anti-50DAG (PA2⁷), anti-43DAG (PA3a^{7,13}), anti-A3b (PA3b²⁰), and anti-laminin antibody (E-Y Inc., San Mateo, CA), were used.

Immunohistochemistry

Sections were prepared as described previously.²³ In brief, after incubation with the primary antibodies (anti-dystrophin, anti-50DAG, anti-43DAG, anti-A3b, anti-35DAG, and anti-laminin), the sections were washed with phosphate-buffered saline. They were then incubated with fluorescein isothiocyanate-labeled goat F(ab')₂ anti-rabbit IgG (Caltag Inc., South San Francisco, CA) in the cases of anti-50DAG, anti-43DAG, and anti-laminin; with fluorescein isothiocyanate-labeled affinity-purified antibody to mouse immunoglobulin G (IgG; Kpl Inc., Gaithersburg, MD) in the cases of anti-dystrophin and anti-35DAG; and with dichlorotriazinyl aminofluorescein-labeled goat F(ab')₂ anti-guinea pig IgG (Chemicon Inc., Temecula, CA) in the case of anti-A3b. After a second washing, the sections were mounted, examined, and photographed.

Immunoblot Analysis

One-dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and two-dimensional PAGE were carried out as described previously.¹⁹ Immunohistochemical staining of proteins was performed as described previously.¹⁹ In brief, after incubation with the primary antibodies (anti-dystrophin, anti-50DAG, anti-43DAG, anti-A3b, and anti-35DAG) and washing, horseradish peroxidase-labeled anti-mouse IgG (Kpl Inc., Gaithersburg, MD) and horseradish peroxidase-labeled anti-rabbit IgG (Cappel Inc., West Chester, PA) were used in the cases of anti-dystrophin and anti-35DAG or anti-50DAG and anti-43DAG, respectively. Biotin-labeled anti-guinea pig IgG (Vector Laboratories, Burlingame, CA) was used in the case of anti-A3b. Finally, the protein on the membrane was visualized with 0.05% diaminobenzidine tetrahydrochloride and 0.035% hydrogen peroxide in 100 mmol/L Tris-HCl.

Before the membrane was incubated with anti-A3b, however, an avidin-biotin blocking kit (Vector Laboratories, Burlingame, CA) was used to prevent endogenous nonspecific staining.

Results

Skeletal Muscle

As shown in Figure 1, anti-A3b (D) homogeneously stained the sarcolemma of control muscle as did anti-dystrophin (A), anti-43DAG (B), anti-50DAG (C), and anti-35DAG (E). On the other hand, anti-A3b (I), anti-50DAG (H), and anti-35DAG (J) hardly stained the

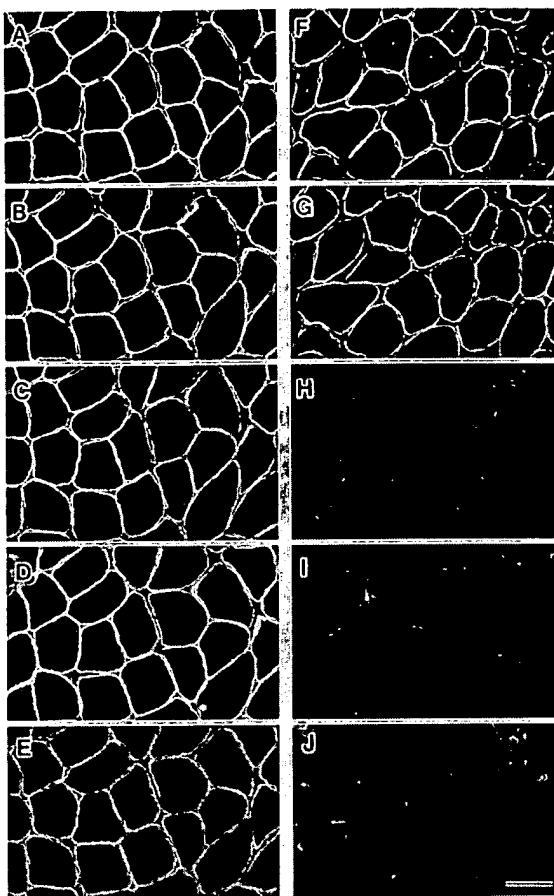


Figure 1. Immunohistochemistry of serial sections of control (A to E) and dystrophic (F to J) skeletal muscles stained with anti-dystrophin (A and F), anti-43DAG (B and G), anti-50DAG (C and H), anti-A3b (D and I), and anti-35DAG (E and J). Control skeletal muscles were positively stained with all antibodies (A to E). Although dystrophic skeletal muscles were stained with an intensity almost equal to that observed in control muscle with anti-dystrophin (F) and anti-43DAG (G), they were hardly stained with anti-50DAG (H), anti-A3b (I), and anti-35DAG (J). Bar = 50 μ .

sarcolemma of dystrophic muscle, whereas anti-dystrophin (F) and anti-43DAG (G) stained it with an intensity similar to that observed in control muscle. Neither control nor dystrophic muscle was stained by the preimmune serum of anti-A3b (data not shown).

When the SDS extract of control muscle separated by SDS-PAGE was stained with anti-A3b (Figure 2), two characteristic bands were detected in addition to the bands assumed to be the endogenous biotin or biotin-binding protein bands that were stained by the preimmune serum. One was an approximately 53-kd band (double arrowhead) and the other was an approximately 42-kd band (single arrowhead). We assume that the 42-kd band corresponds to A3b, as the molecular mass of A3b is similar to that of 43DAG.⁸ Mobility of this band might have been influenced to some extent by the presence of actin, as A3b mi-

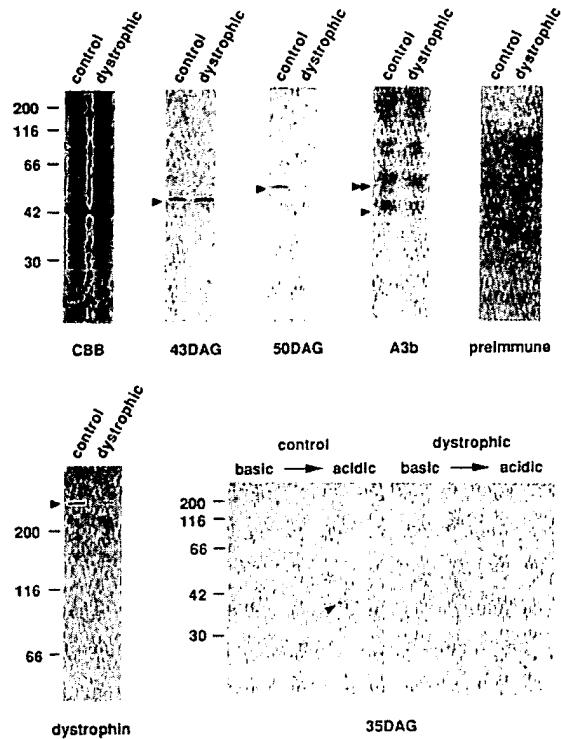


Figure 2. Immunoblot analyses of control and dystrophic skeletal muscles stained with Coomassie brilliant blue, anti-43DAG, anti-50DAG, anti-A3b, preimmune serum of anti-A3b, anti-dystrophin, and anti-35DAG. The samples of control and dystrophic skeletal muscle extracts were placed on the left and right sides, respectively, of each blot. Because it was difficult to detect the 35DAG band with one-dimensional SDS-PAGE, two-dimensional PAGE analysis was used in the case of anti-35DAG.¹⁵ Single arrowhead indicates respective bands or spot. A double arrowhead indicates the band of unknown origin. The positions of molecular markers (kd) are indicated on the left. Coomassie brilliant blue and six antibodies used are indicated at the bottom. A gel containing 6% polyacrylamide was used for the samples stained with anti-dystrophin, and gels containing 10% polyacrylamide were used for the samples stained with Coomassie brilliant blue, anti-50DAG, anti-43DAG, anti-A3b, preimmune serum of A3b, and anti-35DAG. For details see text.

grates in close proximity to actin, which is one of the most abundant proteins in muscle. The quantity of the sample from dystrophic muscle is almost the same as that of the sample from control muscle, judging by the intensity of Coomassie brilliant blue staining. In dystrophic muscle, anti-A3b stained the 53-kd band but not the 42-kd band, corresponding to A3b. As we reported previously,¹⁸ the bands were stained with slightly less intensity with anti-dystrophin and anti-43DAG in dystrophic muscle compared with those in control muscle. However, the band corresponding to 50DAG and the spot corresponding to 35DAG, like the band corresponding to A3b, were not found in dystrophic muscle. The immunohistochemistry and immunoblot analysis demonstrated a selective defect of the sarcoglycan complex.

Cardiac Muscle

As shown in Figure 3, anti-A3b (D) as well as the other antibodies stained the membranes, including T tubules of control muscle, as found in a previous study with anti-dystrophin and other anti-DAPs.^{17,24} Anti-A3b (I), anti-50DAG (H), and anti-35DAG (J) hardly stained the membranes of dystrophic muscle, whereas anti-dystrophin (F) and anti-43DAG (G) clearly stained them. The preimmune serum of anti-A3b did not stain the membranes of control and dystrophic muscles (data not shown). The immunohistochemical findings in cardiac muscle, therefore, were similar to those in skeletal muscle, although the background staining in cardiac muscle was fairly strong.

The results of immunoblot analysis were similar to those for the skeletal muscle (Figure 4). The intensity of the band stained with anti-A3b in cardiac muscle was somewhat greater than that in skeletal muscle.

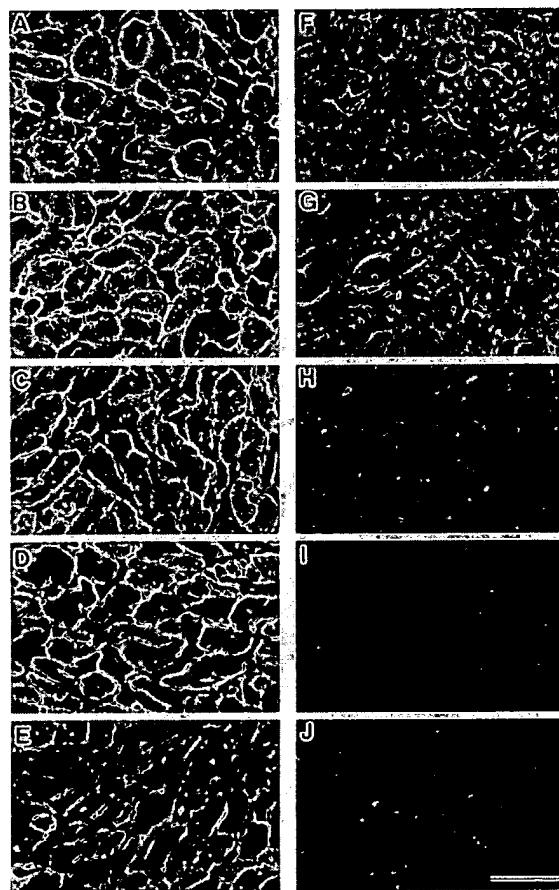


Figure 3. Immunobistochrometry of sections of control (A to E) and dystrophic (F to J) cardiac muscles stained with anti-dystrophin (A and F), anti-43DAG (B and G), anti-50DAG (C and H), anti-A3b (D and I), and anti-35DAG (E and J). For details see text. Bar = 50 μ .

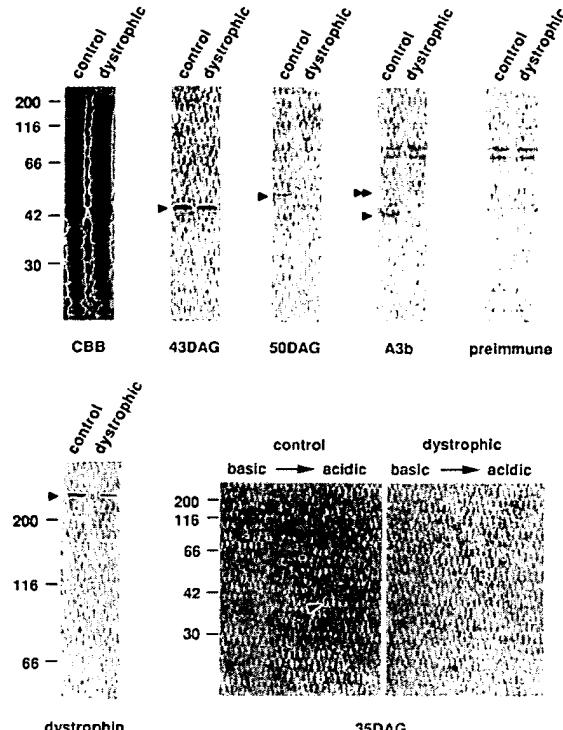


Figure 4. Immunoblot analyses of control and dystrophic cardiac muscles stained with Coomassie brilliant blue, anti-43DAG, anti-50DAG, anti-A3b, preimmune serum of anti-A3b, anti-dystrophin, and anti-35DAG. The placement of samples is the same as that in Figure 2.

Laminin Staining

Anti-laminin clearly stained the sarcolemma of skeletal muscle (Figure 5A, C) and the membranes of cardiac muscle (B, D) in control (A, B) and dystrophic (C, D) hamsters. There was little difference in the results of immunohistochemistry between control and dystrophic muscles.

Discussion

We have shown (Figure 6) that the dystrophin-associated glycoprotein complex binds to the cysteine-rich domain and the first half of the carboxyl-terminal domain of dystrophin⁵ and that these domains bind to 43DAG among the components of glycoprotein complex,²⁵ and we have reported that 43DAG binds to 156DAG.²⁰ As 43DAG has a transmembranous domain in its internal structure and 156DAG binds to laminin, a component of the basement membrane,²¹ it is very plausible that the complex composed of 43DAG and 156DAG, namely, the dystroglycan complex, serves as a link between dystrophin, a subsarcolemmal cytoskeletal protein, and the basement membrane. The sarcoglycan complex,

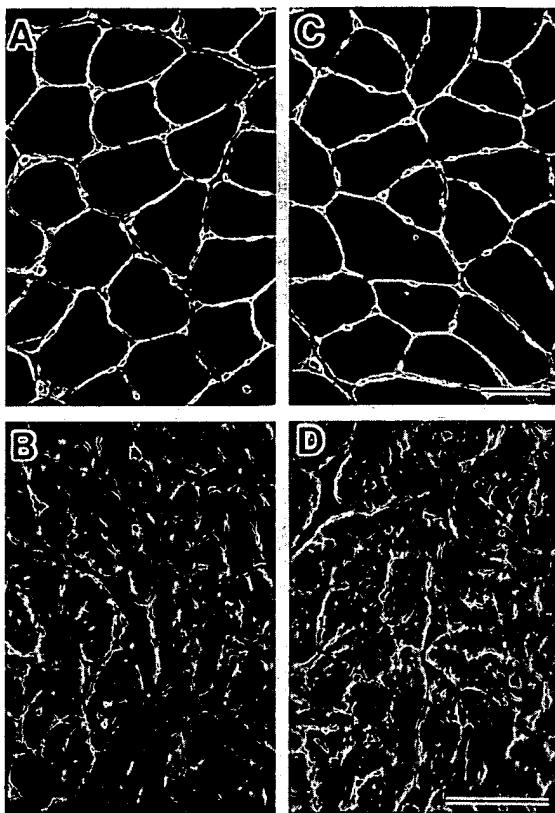


Figure 5. Immunohistochemistry of sections of control skeletal (A) and cardiac (B) muscles and dystrophic skeletal (C) and cardiac (D) muscles stained with anti-laminin. Anti-laminin stained the sarcolemma of control and dystrophic muscles. Bar = 50 μ .

on the other hand, does not appear to have a similar function, because none of the components of the sarcoglycan complex bind to either dystrophin or laminin. Therefore, it is likely that the dystroglycan and sarcoglycan complexes have different functional roles. This is further supported by the difference in tissue distribution of these complexes.^{15,19}

We recently identified A3b as a novel DAP having molecular mass of 43 kd, but being distinct from 43DAG, and showed that A3b is a component of the sarcoglycan complex that, together with the dystroglycan complex, comprises the glycoprotein complex (Figure 6).²⁰ We reported previously¹⁸ that in dystrophic hamster skeletal muscle the levels of 50DAG and 35DAG are greatly reduced whereas 43DAG is present at almost normal levels. These results indicate that some components of a protein group, now known as the sarcoglycan complex, are defective in this muscle, although at that time we were unable to prove this definitively. The introduction of octyl β -D-glucoside into the purified dystrophin-DAP complex enabled us to separate A3b from 43DAG and raise a polyclonal antibody against A3b.²⁰

In the present study, we reinvestigated the skeletal muscle as well as the cardiac muscle of control and dystrophic hamsters with various antibodies, including anti-A3b. The results clearly showed that the sarcoglycan complex is greatly reduced or lost in the striated muscles of the dystrophic hamster, whereas dystrophin, 43DAG, and laminin are well preserved. The dystroglycan complex is also assumed to be preserved, although we did not test for 156DAG, which has already been shown to be preserved in the dystrophic hamster skeletal muscle.¹⁷ Therefore, the linkage via 43DAG and 156DAG between dystrophin and laminin may remain intact,²⁶ even though the sarcoglycan complex is greatly reduced. With the deficiency of the sarcoglycan complex, muscles might degenerate despite the presence of the dystroglycan complex. In other words, merely the presence of the linkage between the subsarcolemmal cytoskeleton and the basement membrane is insufficient to prevent striated muscles from degenerating. Most likely, the sarcoglycan complex plays an essential role in maintaining normal muscle structure and function.

We have found that all three components of the sarcoglycan complex are lost in the dystrophic hamster muscle. However, it is not likely that the disease is due to a simultaneous defect in these three genes; the disease may be caused by a defect in any one of these three genes. When the protein product of the affected gene is abnormal, the sarcoglycan complex might not be formed and thus not be detected by immunochemical methods. Another possibility, which appears to be less likely, is that a defect in an undetermined gene, the protein product of which serves as a *cis* regulatory element that simultaneously regulates the genes of these three proteins, is the cause of the disease. In any case, additional studies are required to clarify these questions.

To accept the dystrophic hamster as an animal model for the SCARMD patient, it is necessary to identify some pathological changes in the dystrophic hamster muscle that are similar to those in SCARMD muscle. SCARMD was first reported to result from a deficiency of 50DAG.¹⁶ Later, it was found that the deficiency of 50DAG is not always the primary cause of all cases of SCARMD. The genetic defect that leads to a 50DAG deficiency in Algerian families is localized to chromosome 13q²⁷ whereas the causative gene is not present in 13q for a Duchenne-like muscular dystrophy in Brazilian families with a 50DAG deficiency.²⁸ Therefore, it is likely that the genetic abnormalities among SCARMD patients are heterogeneous as postulated by Passos-Bueno et al.²⁸ We have found that in SCARMD muscle all components of the sarcoglycan complex is greatly reduced or lost.²⁹ Therefore,

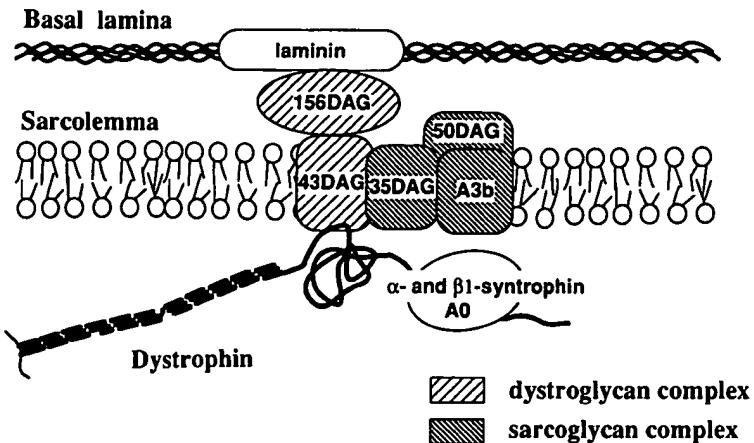


Figure 6. Molecular architecture of dystrophin and dystrophin-associated proteins. (Modified from Suzuki et al²⁵)

whatever the primary cause of SCARMD, the development of the disease may be due to the lack of the sarcoglycan complex. We can thus consider the dystrophic hamster to be a good model for SCARMD, although phenotypical expression of degenerative change is masked in the dystrophic hamster as it is in the case of *mdx* mice even though the *mdx* mice are widely used as the animal model for DMD patient.

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The Receptor Tyrosine Kinase MuSK Is Required for Neuromuscular Junction Formation and Is a Functional Receptor for Agrin

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Intercellular communication is often mediated by receptors on the surface of one cell, which recognize and are activated by specific protein ligands released by other cells. Members of one class of cell-surface receptors, known as receptor tyrosine kinases (RTKs), are characterized by having a cytoplasmic domain containing intrinsic tyrosine kinase activity (Schlessinger and Ullrich 1992). This kinase activity is regulated by the binding of a cognate ligand to the extracellular portion of the receptor. The few RTKs known to be expressed in cell-type-specific fashions have been shown to play roles critical for the growth and differentiation of those cell types. For example, members of the neural-specific trk family of RTKs, which recognize the neurotrophin family of growth factors (Barbacid 1993; Glass and Yancopoulos 1993), are required for the survival and development of discrete neuronal subpopulations (Snider 1994).

We described a receptor-like tyrosine kinase that appears to be restricted to skeletal muscle in its expression, and named it MuSK for muscle-specific kinase (Valenzuela et al. 1995). The mouse MuSK ortholog has been termed Nsk2 (Ganju et al. 1995), and a potential MuSK ortholog has been cloned from the *Torpedo* ray (Jennings et al. 1993).

MuSK protein is detectable in proliferating myoblasts but is dramatically up-regulated as myoblasts exit the cell cycle and fuse to form multinucleated myotubes (Valenzuela et al. 1995). In vivo, innervation by motor axons shortly follows myoblast fusion (Hall and Sanes 1993). Functional innervation depends on the formation of a highly specialized synaptic connection, termed the neuromuscular junction (NMJ), between the incoming motor nerve and the muscle fiber (Hall and Sanes 1993).

The NMJ can be understood as having three distinct components: the presynaptic nerve terminal, where acetylcholine is packaged into synaptic vesicles, and whose end-process is marked by a polarized arrangement of synaptic vesicles and active zones; the postsynaptic motor endplate on the surface of the muscle, a small patch comprising less than 2% of the total surface area of the muscle surface, which is characterized by a dense clustering of particular proteins, some of which may receive nerve-derived signals, as AChRs are known to do, whereas others may be involved in creating the molecular scaffold for this postsynaptic

specialization; and a biochemically distinct protein layer called the "basal lamina," which lies between the nerve terminal and the motor endplate. This basal lamina is distinguished from the adjacent extracellular matrix by the accumulation of a number of proteins, such as acetylcholinesterase and s-laminin; as noted below, the synaptic basal lamina also serves as a reservoir for signaling molecules exchanged between nerve and muscle.

As muscle matures, MuSK becomes highly localized to the motor endplate (Valenzuela et al. 1995). Thus, MuSK is strategically positioned to recognize and mediate a nerve-derived ligand and, in turn, to relay a key signal to muscle. It has long been known that important signals are exchanged across the NMJ (Nitkin et al. 1987; Hall and Sanes 1993; Bowe and Fallon 1995; Burden et al. 1995; Sanes 1995). These signals include the chemical transmitter, acetylcholine, which is released from vesicles in the nerve terminal, recognized by acetylcholine receptors (AChRs) on the muscle, and ultimately results in electrical activation and contraction of the muscle. Muscle also provides neurotrophic factors that support survival of motor neurons (DeChiara et al. 1995), and the nerve may in turn provide myotrophic factors that maintain muscle mass (Helgren et al. 1994). Reciprocal signaling interactions are also critical for both the formation and the maintenance of the NMJ itself. Such signals regulate recognition of nerve-to-muscle contact, arrest the growth of the incoming nerve ending, and induce formation of the highly specialized nerve terminal.

Signals produced by the nerve induce postsynaptic clusters by at least two mechanisms. First, these signals can induce redistribution of preexisting molecules that are initially expressed throughout the myofiber, and second, they can induce localized transcription of specific genes only by subsynaptic nuclei underlying the NMJ. The realization that empty sheaths of the synaptic basal lamina could induce formation of both nerve terminal specializations and motor endplates suggested that key signaling molecules might be embedded in the extracellular matrix (Sanes et al. 1978; Burden et al. 1979; McMahan and Slater 1984; Kuffler 1986).

One nerve-derived factor, embedded in the synaptic basal lamina, is a protein termed agrin, which was discovered for its ability to cause the clustering of pre-

existing AChRs on the surface of cultured myotubes (Godfrey et al. 1984; McMahan 1990; Rupp et al. 1991; Tsim et al. 1992). Agrin has subsequently been shown to co-cluster a number of synaptic components along with AChRs on the surface of cultured myotubes (Wallace 1989). Agrin is a 200-kD protein that can be subdivided into a number of distinct domains. The amino-terminal half contains nine follistatin-like repeats that are homologous to Kazal-type protease inhibitor domains, and the carboxy-terminal half contains four epidermal growth factor (EGF)-like repeats and three regions homologous to the G domains of laminin (Rupp et al. 1991; Tsim et al. 1992). The clustering activity of agrin has been mapped to the carboxy-terminal region; whereas the full-length form of agrin interacts tightly with the extracellular matrix, the carboxy-terminal region can be made in soluble form, and is referred to as "c-agrin" (Ruegg et al. 1992; Tsim et al. 1992; Ferns et al. 1993; Hoch et al. 1994).

A number of different forms of agrin have been discovered, encoded by alternatively spliced transcripts (Ferns et al. 1992; Ruegg et al. 1992; Tsim et al. 1992; Hoch et al. 1993). The carboxy-terminal region of agrin can contain variable insertions at two critical sites, referred to as the Y and Z sites, that can affect the clustering ability of the agrin (Ferns et al. 1992, 1993; Ruegg et al. 1992; Hoch et al. 1994); differential splicing results in transcripts encoding agrins with different combinations of these insertions. Importantly, expression of these different agrin forms is restricted to either muscle or nerve (Ruegg et al. 1992; Hoch et al. 1993). A neuronal-specific insertion at the Z position is absolutely required for the clustering ability of soluble c-agrin, and agrins with an 8-amino-acid insertion at this position are at least 10,000-fold more active than agrins lacking an insert at this position (Ruegg et al. 1992; Ferns et al. 1993). Muscle-derived agrins lack the insertion at the Z position, and thus are not potent clustering agents. A 4-amino-acid insertion at the Y position causes a modest additional increase in the activity of agrins having an insertion at the Z position, and the Y insertion also seems to be involved in the binding of agrin to heparin and proteoglycans (Ferns et al. 1993; Hoch et al. 1994).

A variety of data are consistent with the notion that the *in vitro* clustering actions of agrin reflect an *in vivo* role for agrin in the formation of the NMJ (McMahan 1990). Most important among these are the findings that the highly active forms of agrin are exclusively made by neurons and are deposited in the synaptic basal lamina (Ruegg et al. 1992; Hoch et al. 1993), and that antibodies to agrin block nerve-induced clustering of AChRs on cultured myotubes (Reist et al. 1992). Agrin induces intracellular tyrosine phosphorylations, including that of the β -subunit of the AChR, and inhibitors of tyrosine phosphorylation block agrin-mediated clustering (Wallace et al. 1991; Qu and Huganir 1994; Wallace 1994, 1995; Ferns et al. 1996). Intriguing recent findings have revealed that agrin can

directly bind to an extrinsic peripheral membrane protein, known as α -dystroglycan, via its laminin-like domains (Bowe et al. 1994; Campanelli et al. 1994; Gee et al. 1994; Sugiyama et al. 1994). α -Dystroglycan is part of a protein complex, herein referred to as the dystroglycan complex, that seems to provide crucial anchoring between the extracellular basal lamina and the underlying cytoskeleton (Fallon and Hall 1994). Extrasynaptically, the dystroglycan complex binds laminin in the extracellular matrix and associates with the actin scaffold via a spectrin-like protein known as α -dystrophin, whereas at the synapse, agrin can substitute for laminin and utrophin replaces dystrophin (Fallon and Hall 1994). In addition, the dystroglycan complex is apparently coupled to AChRs by a 43-kD cytoplasmic protein known as rapsyn (Noakes et al. 1993; Apel et al. 1995).

Despite the findings that agrin can bind directly to α -dystroglycan, and that the dystroglycan complex is coupled to AChRs via rapsyn, the role of α -dystroglycan as an agrin receptor remains unclear (Sealock and Froehner 1994). Dystroglycan could be a required component of the functional agrin receptor that is responsible for initiating the signaling response, and it might even be directly involved in activating signaling pathways. However, an agrin mutant has been recently reported that maintains the ability to cluster AChRs without binding to dystroglycan (Gesemann et al. 1996), suggesting that agrin induces clustering via another cell-surface receptor. Here we review recent findings (DeChiara et al. 1996; Glass et al. 1996) demonstrating that agrin acts via a MuSK receptor complex to induce all aspects of NMJ formation.

THE ROLE OF MUSK IN VIVO

MuSK Gene Disruption Results in Perinatal Lethality due to Lack of NMJ Formation

To determine the role of MuSK during skeletal muscle development, we generated mice homozygous for a deletion of an exon encoding the catalytic region of the MuSK tyrosine kinase domain, since such a mutation would abolish MuSK's ability to mediate signals (DeChiara et al. 1996). All the MuSK^{-/-} mice died shortly after birth. Not a single mouse homozygous for the mutation survived the perinatal period. Although normal in their gross anatomy and body weight at birth, the MuSK^{-/-} pups were apparently different in several striking ways from their littermate controls (DeChiara et al. 1996). First, they showed no spontaneous movement and did not respond to a mild tail or leg pinch. Only a strong tail pinch was able to elicit a weak uncoordinated movement. Second, the MuSK^{-/-} pups were cyanotic at birth and appeared not to breathe; histological examination revealed that the lung alveoli of MuSK^{-/-} pups were not expanded, indicating that the pups had never taken a breath.

Neuronal Differentiation and Termination

Given the synaptic localization of MuSK (Valenzuela et al. 1995), we reasoned that the mutant animals' immobility and inability to breathe could be a result of aberrant neuromuscular synapse formation. To determine whether synapse formation is aberrant in MuSK^{-/-} mutant mice, we stained whole mounts of diaphragm muscle with probes that allowed us to assess presynaptic (e.g., synaptophysin antibodies) and postsynaptic (e.g., bungarotoxin to label AChRs) differentiation. In diaphragm from normal animals, nerve processes are restricted to the central region of the muscle, where they terminate shortly after branching from the innervating phrenic nerve. In MuSK^{-/-} mutant mice, synaptophysin-stained nerve processes are not restricted to the central region of diaphragm muscle, and instead are found throughout the muscle. Indeed, synaptophysin-stained nerve processes are nearly as abundant at the ends of the muscle as in the central region (DeChiara et al. 1996). Moreover, the shape of synaptophysin-stained processes in MuSK^{-/-} mutant mice resembles that of preterminal axons rather than that of nerve terminals, and highly arborized nerve terminals are absent in MuSK^{-/-} mice, indicating that MuSK is required for at least some aspects of presynaptic differentiation. Since MuSK is expressed in skeletal muscle and not in motor neurons (Valenzuela et al. 1995), it is likely that the exuberant growth of motor axons and the absence of nerve terminal arborization is due to a lack of MuSK function in muscle and a consequent loss of appropriate retrograde signals that normally prevent continued axon growth and also induce presynaptic differentiation (see Discussion).

AChR clusters, which are found in postsynaptic membrane patches underlying synaptophysin-stained nerve terminals in normal muscle, are absent from MuSK^{-/-} mutant muscle (DeChiara et al. 1996). The absence of AChR clusters in MuSK^{-/-} mutant muscle is not due to a lack of AChR expression, since quantitation using ¹²⁵I-labeled α -bungarotoxin reveals more—albeit diffusely distributed—AChRs in MuSK^{-/-} mutant muscle as compared to normal muscle, and similar numbers of AChRs in cultured MuSK^{-/-} and control myotubes (Glass et al. 1996). Thus, MuSK is required to cluster but not to express AChRs.

The absence of differentiated nerve terminals and AChR clusters in MuSK^{-/-} mutant mice is consistent with the notion that these mice die at birth due to severe abnormalities in both presynaptic and postsynaptic aspects of NMJ formation, and that MuSK is essential for normal formation of neuromuscular synapses *in vivo* (DeChiara et al. 1996).

In addition to the AChRs, approximately one dozen proteins are known to be concentrated in the postsynaptic membrane of neuromuscular synapses, or locally deposited in the overlying synaptic basal lamina, and serve as markers of appropriate postsynaptic dif-

ferentiation (Hall and Sanes 1993; Bowe and Fallon 1995). We examined the distributions of several of these markers to further characterize the extent of postsynaptic deficits in MuSK^{-/-} mice. Although clusters of AChE, ErbB4, utrophin, and rapsyn are all precisely juxtaposed with nerve terminals in sections of muscle from control and MuSK^{+/-} pups, these proteins were concentrated neither at sites stained with antibodies to synaptic vesicle proteins nor anywhere else in muscle from MuSK^{-/-} mutant pups (DeChiara et al. 1996). These results indicate that MuSK was required for every marker of presynaptic, postsynaptic, and basal lamina organization studied.

MuSK^{-/-} Mutant Mice Lack Synapse-specific AChR Gene Expression

In addition to the characteristic localization of proteins presynaptically, postsynaptically, and in the basal lamina, the NMJ junction is notable in that the nuclei underlying the motor endplate (referred to as synaptic nuclei) express a unique set of transcripts. To learn whether MuSK is indeed required for induction of this synapse-specific expression, we used *in situ* hybridization to determine the expression pattern of transcripts encoding the α and δ subunits of the AChR. In control mice, these transcripts are concentrated near myofiber nuclei that are situated near synaptic sites, which appear as a band in the central region of the muscle. In contrast, transcripts encoding both the α and δ subunits are distributed uniformly throughout the myofibers of MuSK^{-/-} mice (DeChiara et al. 1996). Thus, MuSK is required for onset of transcription, and these results suggest that an inability to selectively express AChR genes in synaptic nuclei is responsible, at least in part, for the lack of AChR clustering in muscle from MuSK^{-/-} mice (DeChiara et al. 1996).

THE ROLE OF AGRIN IN MUSK-MEDIATED SIGNALING

Agrin Fails to Induce AChR Clustering in Myotubes Lacking MuSK

The localization of MuSK to the NMJ, together with the absence of NMJs in mice lacking MuSK (MuSK^{-/-} mice), inspired us to ask whether MuSK is required for responsiveness to agrin. Myoblasts isolated from newborn MuSK^{-/-} mice or from control pups were equally able to fuse and form long, twitching myotubes in culture. Together with the observation that skeletal muscle appears rather normal in MuSK^{-/-} mice, these findings indicate that MuSK is not critical for early muscle development and myoblast fusion. On the other hand, MuSK appeared to be absolutely required for AChR clustering *in vitro*, in response to agrin (Glass et al. 1996). After stimulation with the most active form of c-agrin (c-agrin4,8), AChR clusters were evident only in the myotubes from control mice, and not in myotubes from MuSK^{-/-} mice.

Agrin Induces Prominent and Rapid Tyrosine Phosphorylation of MuSK

The inability of agrin to induce AChR clustering in myotubes from $\text{MuSK}^{-/-}$ mice demonstrated that MuSK is required for agrin responsiveness and is consistent with the possibility that MuSK serves as the functional agrin receptor. However, since clustering occurs over a period of hours, these results are also consistent with the possibility that MuSK acts much farther downstream in the agrin signaling pathway. To begin to distinguish between these possibilities, we took advantage of the fact that RTKs become rapidly autophosphorylated on tyrosine upon challenge with their cognate ligand. We decided to assay four of the known forms of soluble agrin, which exhibit differing AChR clustering activities (Ferns et al. 1992, 1993; Ruegg et al. 1992; Hoch et al. 1994), for their ability to induce phosphorylation of the MuSK receptor. Phosphorylation was assessed on the endogenous MuSK receptor that is highly expressed in myotube cultures, obtained by differentiating either the C2C12 mouse myoblast cell line (Valenzuela et al. 1995) or primary rat myoblasts. Strikingly, soluble agrins containing the 8-amino-acid insert at position Z (c-agrin4,8 and c-agrin0,8), which are the forms capable of inducing AChR clustering, were also the forms that induced prominent tyrosine phosphorylation of MuSK (Glass et al. 1996). The agrin most active in clustering (c-agrin4,8) was also most active in inducing MuSK phosphorylation. In contrast, the soluble agrins lacking the 8-amino-acid insert (c-agrin4,0 and c-agrin0,0), which cannot induce AChR clustering, also could not induce MuSK phosphorylation.

The activation of a RTK by its cognate ligand typically tends to occur rapidly, and we could demonstrate that agrin induces tyrosine phosphorylation of MuSK with kinetics similar to those seen for well-characterized RTK/ligand systems; induction was detectable by 1 minute, peaked within the first 5 minutes, and remained elevated for over an hour (Glass et al. 1996). The tyrosine phosphorylation of MuSK also occurred using agrin at concentrations similar to those noted for other ligands that act on RTKs (Ip et al. 1993), with phosphorylation detectable using 1 nM agrin (Glass et al. 1996).

The requirement of MuSK for agrin responsiveness, the ability of agrin to induce rapid and prominent MuSK phosphorylation, the specificity of agrin for MuSK as compared to other factors tested, and the precise correlation of agrin forms active in AChR clustering assays and in MuSK phosphorylation assays together continued to support the notion that MuSK serves as the functional agrin receptor.

Agrin Does Not Directly Bind to an Isolated MuSK Ectodomain

If MuSK is indeed the functional agrin receptor, we would have expected to be able to demonstrate binding

of agrin to MuSK. In an attempt to demonstrate such binding, we first constructed an expression construct encoding a fusion protein between the ectodomain of rat MuSK and the Fc portion of human immunoglobulin G1 (designated MuSK-Fc). Similar receptor-Fc fusions have previously been used to characterize binding between RTKs and their ligands (see, e.g., Davis et al. 1994; Stitt et al. 1995). We tried to demonstrate binding of MuSK and agrin *ex vivo* through a variety of methods, including simply by attempting to use MuSK-Fc to detect agrin immobilized onto nitrocellulose. In contrast to our control experiments, in which immobilized brain-derived neurotrophic factor (BDNF) was easily detected by an Fc fusion of its cognate receptor (TrkB-Fc), and in which immobilized agrin was easily detected by the agrin-specific monoclonal antibody, immobilized agrin could not be detected by MuSK-Fc (Glass et al. 1996). All other methods undertaken to get direct binding of MuSK-Fc to agrin *ex vivo* were similarly unsuccessful (Glass et al. 1996).

The negative binding results showed that the isolated MuSK receptor was not sufficient to bind agrin. Thus, despite much functional data indicating that agrin acts via MuSK, the possibility was raised that MuSK may not directly serve as a receptor for agrin. Alternatively, MuSK may require additional components or modifications to bind and respond to agrin.

Agrin Activates MuSK in a Cell-context-dependent Fashion

Seeing the results described above, we considered the possibility that the agrin-MuSK interaction required additional components. We reasoned that if an accessory component was required, it might be specifically expressed only on cells normally responding to agrin. Thus, we ectopically expressed full-length cDNAs encoding rat, human, and chicken MuSK in fibroblasts and assayed whether these MuSK receptors could be inducibly phosphorylated by agrin. When expressed in fibroblasts or undifferentiated myoblasts, the three transfected species of MuSK could not be phosphorylated in response to agrin (Glass et al. 1996).

When transfected myoblasts were differentiated into myotubes, however, the introduced MuSK receptors were as effectively activated by agrin as was the endogenous mouse MuSK (Glass et al. 1996). Both introduced and endogenous MuSK had identical profiles of responsiveness to the various forms of agrins, with activations mediated only by forms having the 8-amino-acid insert at the Z position. Thus, our cDNAs encode MuSK proteins that are perfectly competent to undergo agrin-induced phosphorylation, but they can only be activated by agrin in the context of a differentiated myotube, consistent with the notion that agrin activation of MuSK requires a myotube-specific accessory component that is not expressed in fibroblasts or undifferentiated myoblasts.

A Receptor Complex Can Be Demonstrated between Agrin, MuSK, and a Myotube-associated Specificity Component

Altogether, the data indicate that agrin requires MuSK to mediate clustering and that agrin activates MuSK very rapidly, but that agrin does not directly bind to a purified MuSK ectodomain and can only activate MuSK in the context of a myotube. These findings are consistent with the possibility that MuSK is a requisite part of an agrin receptor complex, but that although MuSK provides a key signaling function for this complex, it requires another component(s) to bind to agrin. Similar types of receptor complexes have been described for other ligands. Perhaps some of the best-characterized examples include the receptor complexes for ciliary neurotrophic factor (CNTF) and its cytokine relatives (Davis et al. 1993; Stahl and Yancopoulos 1993). In order to interact with its two signal transducing β receptor components, gp130 and LIFR β , CNTF must first bind to its α receptor component, known as CNTFR α . CNTFR α serves no signaling role, and is in fact linked to the surface via a glycosylphosphatidyl-inositol linkage and thus has no cytoplasmic domain. The receptor complex for CNTF is built in step-wise fashion: CNTF first binds to CNTFR α ; this initial complex can then bind to and recruit a single β component; finally, a complete complex forms that involves β component dimerization, which is required for signal initiation. In the final complex, CNTF seems to make contacts with all three receptor components, and can be directly cross-linked to all three components (Stahl and Yancopoulos 1993). Interestingly, receptor complexes for CNTF can be built in solution using just the soluble ecto-domains of the various components. Furthermore, if just one of the receptor components is linked to the surface, a receptor complex can be built around it using soluble versions of the other components, but only in a CNTF-dependent fashion.

To confirm that MuSK directly interacts with agrin as part of its receptor complex, we next demonstrated that radiolabeled agrin could be cross-linked to MuSK receptors on the surface of myotubes. Immunoprecipitations using a MuSK-specific antibody, from lysates of myotubes chemically cross-linked to radiolabeled recombinant human agrin, contained complexes corresponding in size to agrin/MuSK complexes. These agrin/MuSK complexes were not seen in the presence of excess unlabeled agrin, or if a peptide was used to block MuSK precipitation.

Since agrin appears to bind MuSK directly in a receptor complex, we reasoned that we might be able to manipulate this complex in much the same way the CNTF receptor complex can be manipulated. To confirm the possibility that myotubes specifically express an accessory component(s) required for agrin to bind MuSK, we decided to test whether we could specifically build a receptor complex on the surface of myotubes, but not on other cells, using agrin together with a soluble version of the MuSK receptor to complex to

the putative accessory component(s) on the surface of myotubes. Confirming this possibility, we found that soluble MuSK-Fc can be directly cross-linked to radiolabeled agrin, but only in the presence of membranes from differentiated myotubes, and not with membranes from fibroblasts (T.N. Stitt et al., unpubl.). These data demonstrate that complexes can form between agrin and MuSK, but only in the presence of a myotube-associated specificity component(s), which we call MASC. Potential ternary complexes were identified that might contain agrin, MuSK, and MASC.

DISCUSSION

Whereas early skeletal muscle development apparently proceeds rather normally in MuSK $^{-/-}$ mice, every aspect of NMJ formation that we examined is absent in these mice (DeChiara et al. 1996). Branches of the main intramuscular nerve do not establish normal contacts with the muscle, do not form correctly positioned or specialized nerve terminals, and are apparently not given appropriate signals to stop their wandering aimlessly across the muscle. Furthermore, we found no evidence of postsynaptic differentiation, since muscle-derived proteins that are normally localized to the synaptic basal lamina or the postsynaptic membrane are instead uniformly distributed in MuSK $^{-/-}$ myofibers. The defects in NMJ formation are sufficient to account for the perinatal lethality of the MuSK $^{-/-}$ mice resulting from their inability to breathe, and for their immobility.

Because of the absence of NMJs in mice lacking the MuSK RTK, we proceeded to test the possibility that agrin acts via the MuSK receptor and demonstrated that MuSK indeed serves as a required signaling component of a complex receptor that specifically responds to the neural forms of agrin (Glass et al. 1996). Together, our findings indicate that agrin, acting via the MuSK receptor, is required for all aspects of NMJ formation.

The defects in NMJ formation in mice lacking MuSK are more profound than might have been predicted, based on the in vitro actions of agrin, for mice lacking a functional agrin receptor. The previously described in vitro actions of agrin are largely limited to its organizing effects on postsynaptic differentiation (McMahan 1990; Hall and Sanes 1993; Bowe and Fallon 1995). The aberrant behavior of the preterminal axons and the lack of synapse-specific transcription represent deficits that would not necessarily have been expected to occur in mice in which agrin-mediated signaling pathways had been disrupted. However, recent generation of mice lacking agrin reveals NMJ defects that are indeed as profound as those described here for mice lacking MuSK (Gautam et al. 1996), consistent with the idea that MuSK is a required component of the functional receptor for agrin. Since MuSK is located only on the postsynaptic side of the NMJ (Valenzuela et al. 1995), it seems likely that the aberrant be-

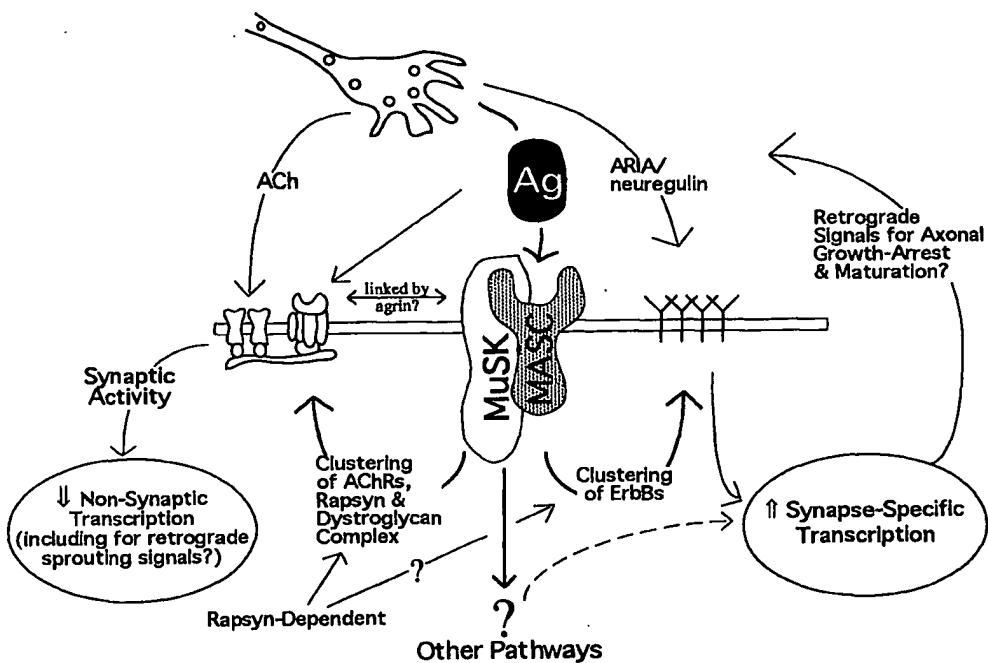


Figure 1. Schematic representation of role played by the agrin/MuSK/MASC signaling system. MuSK appears to activate signaling cascades that are responsible for all aspects of NMJ formation, including postsynaptic organizations, synapse-specific transcription; and presynaptic growth and differentiation (perhaps by regulating the elaboration of retrograde signals, or simply by promoting synaptic activity that down-regulates production of sprouting factors). MuSK is diagrammed as being recruited after agrin binds MASC, since agrin does not bind directly to MuSK ex vivo. MuSK activates at least two independent pathways, one that is rapsyn-dependent and leads to AChR and dystroglycan clustering, and one that appears to be rapsyn-independent and results in synapse-specific transcription. In the model depicted, synapse-specific transcription is presumed to involve clustering of erbB receptors. However, it has recently been shown that synapse-specific transcription occurs in rapsyn-deficient mice although they lack clustered erbB3 receptors (Moscoso et al. 1995), indicating that synapse-specific expression does not require clustering of erbB3 in particular; it may still involve clustering of other erbB receptors (e.g., erbB4), or alternatively, it may depend on erbB-independent pathways. (Modified, with permission, from DeChiara et al. 1996 [copyright Cell Press].)

havior of the presynaptic terminals in $\text{MuSK}^{-/-}$ mutant mice is due to indirect actions of the agrin/MuSK signaling system (Fig. 1). Thus, we favor the idea that agrin released from the nerve terminal causes the post-synaptic muscle cell, via MuSK activation, to reciprocally release a recognition signal back to the nerve (Fig. 1) to indicate that a functional contact has occurred. In response to this muscle-derived recognition signal, the nerve stops growing and undergoes presynaptic differentiation; the ability of basal lamina sheaths to induce presynaptic differentiation in the absence of the underlying muscle suggests that muscle-derived signals may be embedded in synaptic basal lamina, like agrin and neuregulin (Sanes et al. 1978). Alternatively, we cannot eliminate the possibility that excessive neuronal growth in $\text{MuSK}^{-/-}$ mutant mice is caused by persistent production of a muscle-derived sprouting factor that is normally down-regulated following functional innervation (Brown et al. 1981), or by the absence of appropriate synaptic activity (Balice-Gordon and Lichtman 1994). It should be noted that despite the defects displayed by nerve branches, the main intramuscular nerves appear to be properly positioned within the muscle in $\text{MuSK}^{-/-}$ mice, indicating

that guidance mechanisms which bring motor axons to muscle remain intact and operate independently of the agrin/MuSK signaling system.

As with the deficits in presynaptic differentiation, it seems likely that the lack of synapse-specific transcription in $\text{MuSK}^{-/-}$ mice also results from an indirect mechanism (Fig. 1). That is, although AChR genes are expressed uniformly in muscle fibers from $\text{MuSK}^{-/-}$ mice, we do not favor the idea that MuSK is the receptor for the extracellular signal that activates synapse-specific transcription. At present, the best candidate for such a signal is neuregulin, acting via the erbB3 and/or erbB4 receptors localized in the postsynaptic membrane (Altiock et al. 1995; Moscoso et al. 1995; Zhu et al. 1995). Thus, it seems likely that the loss of synapse-specific transcription is another indirect by-product of the fact that disruption of agrin/MuSK signaling blocks normal synaptic differentiation, resulting in an inability of the muscle cell to cluster erbB receptors and possibly an inability of mutant nerve terminals to provide neuregulin.

Despite our findings, the role of dystroglycan as an agrin receptor remains unclear. The comparison of $\text{MuSK}^{-/-}$ and $\text{agrin}^{-/-}$ mutant mice, together with the

signaling data for MuSK, indicate that MuSK is the critical receptor component that initiates agrin-mediated signaling pathways. Recent evidence demonstrates that a 27-kD protein fragment of agrin is sufficient to induce AChR clustering and yet does not bind dystroglycan at all (Gesemann et al. 1996), casts doubt on any functional role for dystroglycan in agrin-mediated phosphorylation or clustering.

The essential role of MuSK at the NMJ raises the possibility that MuSK or related RTKs are playing similar roles in other synapses, particularly since low levels of MuSK mRNA are detectable in the central nervous system (Valenzuela et al. 1995; and data not shown). Agrin, as well as binding sites for agrin, has been detected in the central nervous system (Bowe and Fallon 1995), suggesting that analysis of synapses in the central nervous systems of MuSK^{-/-} mice is warranted. In this regard, a pair of receptors known as the RORs (Masiakowski and Carroll 1992), which are expressed during early development of the central nervous system and whose closest relative is MuSK, should probably also be evaluated for roles in synapse formation. In any case, the complex types of spatial organizing events initiated by MuSK have not previously been noted in response to other RTKs, which have instead been largely characterized for their ability to mediate responses to growth factors that elicit mitogenic, survival, or differentiative actions (Schlessinger and Ullrich 1992). It remains possible that, in addition to its organizing role at the NMJ, the agrin/MuSK system is also involved in more classic growth-factor-like responses.

MuSK Is a Requisite Subunit of the Agrin Receptor Complex but Requires a Myotube-associated Specificity Component

Our data have indicated that MuSK is a requisite subunit of a multicomponent receptor complex used by agrin and that it requires a myotube-specific accessory component(s) to bind and mediate responses to agrin. There are many other examples of ligands that use multicomponent receptor complexes (Stahl and Yanopoulos 1993). Most cytokines, as noted above for CNTF and its relatives, use such receptor complexes. In these receptor complexes, there are often components whose primary role is to bind ligand, whereas other subunits can both bind and signal. In the case of RTKs, there are also examples of multicomponent receptors. For example, fibroblast growth factors (FGFs) need to bind heparin, usually presented on surface proteoglycans, in order to bind and activate their RTK subunits (Goldfarb 1990). The trk family of RTKs, which bind to nerve growth factor (NGF) and the related neurotrophins, present a less well-understood example of RTKs which may interact with an accessory component known as p75 (Barbacid 1993). In the case of ARIA/neuregulin, receptor complexes can include two different members of the ErbB

family (Burden et al. 1995). Most recently, and most analogous to MuSK/MASC, it has been shown that binding and activation of the ret RTK by glial cell-line-derived neurotrophic factor (GDNF) requires an accessory component termed GDNFR α (Jing et al. 1996; Treanor et al. 1996). In all these examples of multi-component receptor complexes, signal initiation seems to depend on ligand-mediated dimerization of signal-transducing subunits that are included within the receptor complex; this dimerization can involve either homodimerization of identical subunits or heterodimerization of related subunits.

Before considering potential candidates for the other component(s) of the agrin receptor complex, it is worth remembering that the signaling function of MuSK appears quite unusual as compared to other RTKs. Whereas most RTKs are thought to mediate growth or survival responses, or quite simple differentiative events, MuSK seems instead to mediate a very complicated organizing function. The structure of the NMJ has already been shown to depend on complex molecular scaffolds that anchor components in place by linking them to the extracellular matrix as well as to the cytoskeleton. Since they combine to initiate formation of the entire postsynaptic macrostructure, and also are contained within it, it seems likely that both agrin and MuSK have many interactions with molecules at the NMJ, for both structural and signaling purposes. One can imagine that MuSK interacts with proteins that serve to link it to the molecular scaffold at the junction, both extracellularly and intracellularly; it may bind to other proteins that are direct substrates for its kinase activity or that act merely as couplers of MuSK to its substrates. Thus, the myotube-specific accessory component(s) required for agrin to bind and activate MuSK may be difficult to distinguish from other (perhaps more abundant) molecules interacting with agrin and MuSK that serve other functions; even the accessory component(s) itself may be multifunctional, and not only serve to help bind agrin, but to couple this ligand/receptor system to other proteins as well.

There are clues concerning the possible identity of accessory receptor components for agrin. Recent data implicate a synapse-specific carbohydrate in agrin responses (Martin and Sanes 1995); enzymatic removal of this carbohydrate blocks agrin responsiveness, whereas lectin-mediated clustering of proteins with this carbohydrate, or enzymatic unmasking of more of this carbohydrate, potentiate or even mimic agrin. These data could be reconciled with the concept of a MuSK receptor complex if such a carbohydrate marked and was required for agrin recognition by the MuSK accessory component. Alternatively, it could be that such a carbohydrate is required on MuSK itself. Along these lines, it should be pointed out that a myotube-specific modification of MuSK, whether it involves carbohydrate or other modifications, may allow MuSK to directly bind agrin, and thus raises the possibility that modified MuSK itself might correspond to the

myotube-specific "accessory" component; in this case, soluble recombinant MuSK would presumably be able to dimerize with the correctly modified MuSK—via agrin—on the myotube surface. A related scenario might involve the expression of a MuSK relative in myotubes, and the need for heterodimerization between MuSK and this relative—as can occur with the ErbBs—to create a functional agrin receptor. Finally, since agrin forms lacking the ability to bind heparin are still active, it seems unlikely that binding to a proteoglycan at the "Y" site would be critical for linking agrin to its accessory component.

Mechanism by Which MuSK Activation Triggers Postsynaptic Organization

Previous studies had implicated tyrosine phosphorylation in the agrin response (Wallace et al. 1991; Wallace 1994, 1995; Qu and Huganir 1994; Ferns et al. 1996). The finding that the critical signaling component of the agrin receptor complex is a RTK validates the notion that specific agrin-induced tyrosine phosphorylations initiate organization and formation of the NMJ. The only well-characterized agrin-induced tyrosine phosphorylation, other than that of MuSK, is of the AChR β component (Wallace et al. 1991; Qu and Huganir 1994; Ferns et al. 1996). As would be expected, although phosphorylation of AChR β is relatively rapid and begins by 15 minutes after agrin addition, it trails MuSK phosphorylation. It is unknown whether AChR β phosphorylation is required for, or contributes to, AChR clustering, or whether it is a direct substrate of MuSK. The critical MuSK-mediated phosphorylations may well involve proteins that are clustered or directly involved in building the molecular scaffold that maintains the postsynaptic specialization. It is easy to imagine that direct phosphorylation of key proteins may trigger complexes or aggregations to form; for example, AChR β phosphorylation may trigger its association and co-aggregation with rapsyn or other coupling proteins. Since MuSK appears to be much less abundant than many of the proteins that are clustered, it would presumably act catalytically on such structural targets. Alternatively, MuSK-mediated phosphorylations may not directly trigger clustering events, but rather, MuSK may activate downstream enzymes or signaling pathways that in turn trigger clustering. These possibilities emphasize the need to identify direct substrates and signaling pathways activated by MuSK.

MuSK Signaling in Other Contexts: In the Adult, Atrophies and Dystrophies?

The complex type of organizational role played by MuSK at the NMJ distinguishes it from other growth factors that use RTKs. However, this unusual role does not preclude MuSK from having more traditional growth-factor-like effects. Exploration of the signaling

pathways activated by MuSK will certainly help address whether MuSK uses unique signaling pathways or instead shares pathways with other RTKs. When it was realized that the trk family of RTKs served as the receptors for NGF and its relatives, it was thought that the trks might prove to have unusual substrates because they mediated neuronal survival and differentiation responses, as opposed to the conventional type of proliferative responses that had previously been attributed to RTKs. It turns out, instead, that the trks activate much the same substrates as mitogenic RTKs, but that these activations are ultimately interpreted much differently within the context of a postmitotic neuron (Glass and Yancopoulos 1993). In fact, ectopic expression of the trks in proliferation-competent cells, such as fibroblasts, allows them to mediate conventional mitogenic responses indistinguishable from those of FGFs or EGFs (Glass et al. 1991). Thus, it will be important to determine whether the unusual actions of MuSK are a consequence of unique signaling capabilities, or simply of its unique site of expression.

Current studies of agrin and MuSK have almost completely focused on their roles during formation of the NMJ. In the entirely different context of an adult muscle, MuSK is dramatically up-regulated in situations where the muscle is at risk for atrophy, including during forced immobilization (Valenzuela et al. 1995). It will certainly be of interest to determine what kinds of actions MuSK mediates in the context of an adult muscle, whether these are more along the trophic types more usual for a RTK, and whether these actions would be of benefit during muscle atrophy or other muscle diseases. Along these lines, it is worth noting that MuSK, via agrin, now appears to be linked to the dystroglycan complex. Mutations in at least three of the components of this complex (dystrophin, emerin, and adhalin) account for different types of muscular dystrophies (Campbell 1995). The mechanism by which problems in the dystroglycan complex lead to progressive muscle wasting are poorly understood, and efforts to understand this mechanism are primarily focused on problems with sarcolemmal stability. The association of a RTK with the dystroglycan complex would certainly raise the possibility that signaling defects play an important contributory role in dystrophies.

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